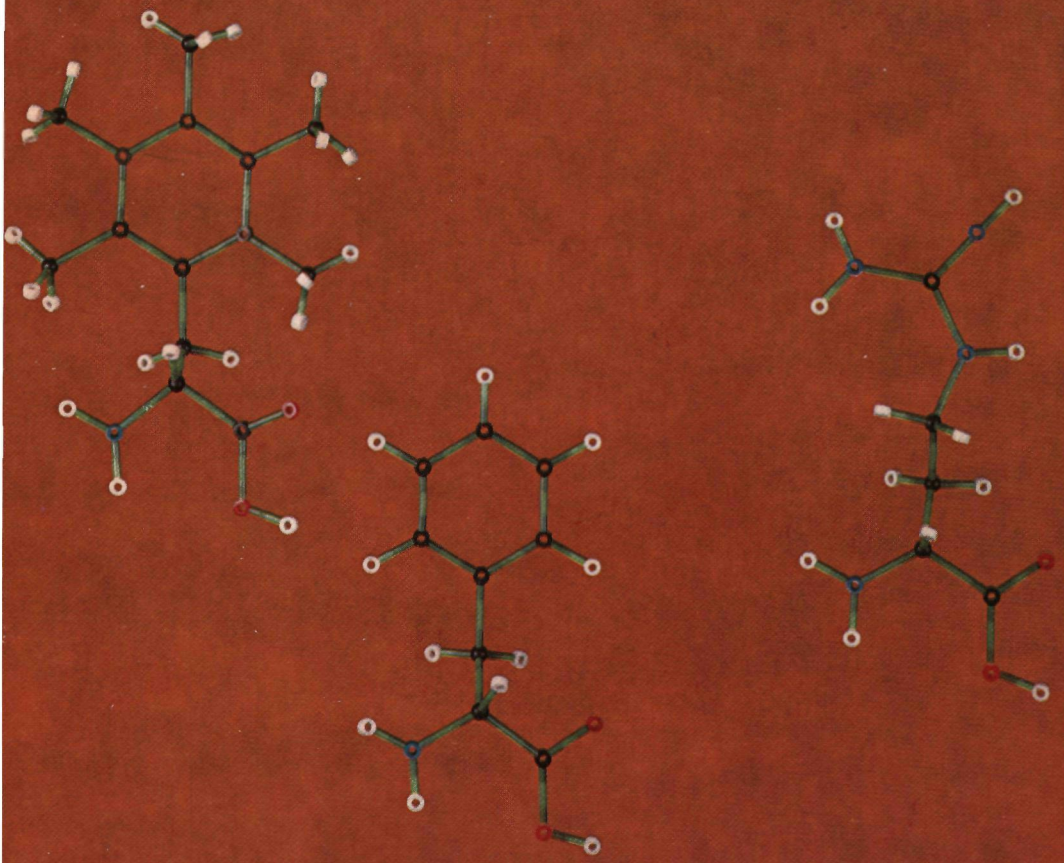
SYNTHESIS AND PROPERTIES OF SOME ACTH AND α-MSH ANALOGUES

STRUCTURE-FUNCTION RELATION



J. W. F. M. van NISPEN

SYNTHESIS AND PROPERTIES OF SOME ACTH AND α-MSH ANALOGUES

STRUCTURE-FUNCTION RELATION

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SYNTHESIS AND PROPERTIES OF SOME ACTH AND α-MSH ANALOGUES

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
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CHAPTER VIII

C H A P T E R I

GENERAL INTRODUCTION

I. 1 ACTH AND α-MSH, OCCURRENCE, BIOLOGICAL ACTIVITY AND STRUCTURE

I. 1.1 ACTH

The ACTH molecule is secreted by the basophilic β'-cells of the anterior pituitary gland (adenohypophysis) and reaches the adrenals *via* the bloodstream. Its principal activity is the stimulation of the adrenal cortex to produce and release steroid hormones (chiefly cortisol, cortisone and corticosterone). The most important extra-adrenal activities involve lypolytic and melanotrophic effects.

The hormone appears to be a single-chain polypeptide, composed of 39 amino acid residues. The *C*-terminal portion of the hormone, consisting of the last 15 amino acid residues, appears to be *species*-specific, which suggests that this fragment is not essential for eliciting the biological response. The revised structure of human ACTH, recently reported by Riniker *et al.*[1] is given in fig. I, 1.

As a result of studies which were initiated around 1960 by several groups[2,3], concerning the synthesis and biological activities of partial

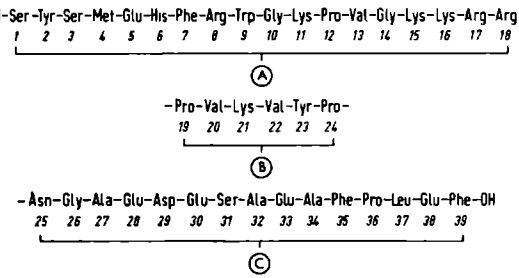


Fig. I, 1

ACTH sequences, the peptide hormone can be divided into three parts, A, B and C. Fragment C (sequence 25-39) is the *species*-specific part, contributing to the immunological specificity. The remaining chain 1-24 (parts A and B) possesses full biological activity. The sequence 1-18 (part A) represents the shortest fragment of the molecule which displays full biological activity, provided that a *C-terminal amide function is present*, as has been proven by biological tests employing synthetic samples of this fragment[3,4].

Many ACTH analogues, containing 18 to 24 amino acid residues, have been synthesized for structure-activity relationship studies. The results suggest the existence of independent *loci* with different functions. Hofmann and co-workers distinguished an "active site" and a binding site [2,5,6]. The information-bearing active site appeared to be located in the sequence 1-10 and was confined to fragment II (sequence 5-10) because

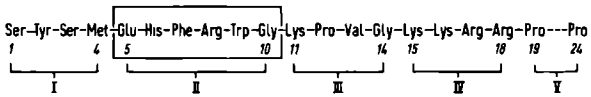


Fig. I, 2

L-methionine[4] seemed to have more of a binding function (fig. I, 2). They suggested that fragment III (sequence 11-14) was the binding site for melanotrophic activity whereas fragment IV (sequence 15-18) in combination with III had the same function in respect of adrenocorticotrophic activity.

The most far-reaching attribution of functions to regions of the ß-corticotrophin-(1-24)-tetracosapeptide* was presented by Schwyzer and co-workers [7,8,9]. Schwyzer suggested a "sychnologic" composition of the hormone which means that functional elements ("words" composed of combinations of amino acids) are arranged in an uninterrupted way. This contrasts with a "rhegnylogic" composition, as in insulin, which contains discontinuous words *i.e.* the elements composing these words are separated by biologically less relevant peptide regions. Sychnologic hormones are always flexible, consist of a straight chain and are easily bound to their receptors; rhegnylogic hormones should be inflexi-

* Nomenclature of Bell, H.P. (1954) *J. Amer. Chem. Soc.* 76, 5565-5567. According to Li, C.H. (1959) *Science* 129, 969-970, the compound should be named α-adrenocorticotrophin.

ble[8]. According to Schwyzer, receptors recognize a hormone by its "address", and receive a stimulus from its "message". In ACTH the message is present in the 5-10 sequence (fragment II) and the address in the (11-24) tetradecapeptide (fragment III + IV + V) which behaves as a competitive antagonist[9]. Fragment I functions as the word for "potentiation".

I. 1.2 α-MSH

The skin-darkening effect of α-MSH and β-MSH, two melanocyte-stimulating hormones isolated from the pituitary glands of several organisms, was discovered independently by Smith[10] and Allen[11].

α-MSH is a straight-chain tridecapeptide with *N*-acetyl-serine as the *N*-terminal and valine amide as the *C*-terminal. The amino acid sequence is identical with that of the *N*-terminal tridecapeptide of natural ACTH (see fig. I, 1, 2). It has the same composition regardless of the source.

It has been suggested that the fragment -Met-Glu-His-Phe-Arg-Trp-Gly-, comprising the sequence 4-10 in ACTH, α-MSH and β-MSH, is responsible for the physiological effect. Li found that a synthetic sample of this heptapeptide had about 60% of the activity of α-MSH[12]. The smallest fragment which possessed weak melanocyte-expanding activity appeared to be the tetrapeptide H-His-Phe-Arg-Trp-OH[13]; apparently it contains the "active site" for melanocyte stimulation. Extension of the

peptide chain from either the amino or carboxyl terminus increases biological activity, which appears to reach its maximum at a chain-length of 13 to 15 amino acid residues and which declines again on further elongation.

Every peptide known to date that possesses adrenocorticotrophic activity has also the ability to expand melanocytes, although the converse is not necessarily true; α-MSH itself exhibits only a weak adrenal cortical activity[14,15]. In view of the close parallelism of these two types of biological activities, it is not surprising to find that amino acid substitutions which affect one of these biological functions also alter the other. Substitutions that enhance steroidogenic activity also enhance melanocyte-stimulating activity. Until now all alterations that lower adrenocorticotrophic activity have brought about a proportional lowering of the melanocyte-stimulating activity[16].

I. 2 SUBSTITUTIONS AT THE "ACTIVE SITE" OF ACTH AND α-MSH

I. 2.1 Arginine[8]

A first objective of the structure-activity relationship investigations made in our laboratory concerned the elucidation of the role of the basic amino acids. In order to investigate this all the basic amino acid residues in fragment A were systematically replaced by L-ornithine residues. Some

of these analogues were investigated with L- as well as D-serine at position 1 [17,18,19]. Replacement of Lys[11,15,16] and Arg[17,18] appeared to be allowed, and in some cases even resulted in a prolongation of biological activity. Replacement of Arg[8] by Orn, however, caused almost total loss of activity[18], a similar result being found when Arg[8] was substituted by L-lysine[20]. These observations point to the particular significance of the guanidino group of the arginine residue in this position. The influence of the length of the side-chain of Arg[8] was then investigated by synthesizing[21] and testing[22] ACTH analogues containing the higher homologue of arginine, L-homoarginine, in position 8. These peptides showed a surprisingly high degree of biological activity: in all the tests performed *viz.* steroidogenesis *in vivo* and *in vitro* and lipolysis *in vivo* and *in vitro*, the loss of activity due to the substitution did not exceed a factor of about 4[22].

In view of these results we have now synthesized similar ACTH peptides containing the lower homologue of arginine, L-norarginine* (L-α-amino-γ-guanidinobutyric acid) in position 8, in order to investigate whether shortening of the arginine side-chain with a methylene group again results in a product which still fulfils the demands for ac-

* By analogy with the abbreviation Har for L-homoarginine, we will use the three-letter symbol Nar for L-norarginine.

tivating the receptor.

I. 2.2 Tryptophan[9]

As early as 1957 Dedman *et al.* stated that the presence of a tryptophan residue at position 9 in ACTH was probably essential for biological activity[23]. In order to obtain more information concerning the importance of the Trp^{9} residue for hormonal activity, Hofmann *et al.* investigated the effect of replacement of tryptophan by phenylalanine. For [Gln^{5}, Phe^{9}]-ß-corticotrophin-(1-20)-amide[24] he found a nearly complete loss of steroidogenic activity; the melanocyte-stimulating activity of the compound appeared to be about 10^{7}U/g (about 10^{8}U/g for ACTH-(1-24)). Further investigations using a particulate fraction from beef adrenal cortical tissue which appeared to contain ACTH receptors[5] led to the conclusion that the tryptophan residue in ACTH had an, as yet undefined, "functional" importance in the very process by which ACTH exerts its physiological action[16].

The replacement of tryptophan by N^{α}-methyltryptophan is another substitution that eliminates adrenocorticotrophic activity almost completely[25].

The most recent communication concerning the role of tryptophan in ACTH comes from Ramachandran *et al.*. Chemical modification of the single tryptophan residue in ACTH by reaction with *o*-nitro-

phenyl sulphenyl chloride results in the loss of the lipolytic activity of the hormone in rat fat cells[26]. On the other hand, Nps-ACTH was more potent than ACTH in stimulating lipolysis in rabbit fat cells and in darkening amphibian skin[27]. The steroidogenic potency of the modified peptide was reduced to about 1/70-1/80[28].

Seelig, Kumar and Sayers have tested [Trp(Nps)^{9}]-β-corticotrophin-(1-39) and [Trp(Nps)^{9}]-β-corticotrophin-(1-24) in the isolated rat adrenal cell system of Sayers *et al.*[29] and found that the maximum rate of steroidogenesis is less than that induced by the agonists ACTH-(1-39) and ACTH-(1-24). Their conclusion was that these observations emphasize the importance of unmodified tryptophan residue at position 9 in ACTH in the process involved in the excitation of the receptor[30].

Up to now, replacement of tryptophan^{9} in α-MSH by another amino acid has not been investigated. Substitution of L-tryptophan by its D-isomer, carried out in the pentapeptide sequence 6-10, resulted in a compound with a slightly lowered activity according to Yajima[31]. Koida, however, found the modified peptide to be 17 times more potent than the all-L-peptide[32]. Similar pentapeptides with several other combinations of L- and D-amino acids have been synthesized and tested; the activities found vary widely[16]. α-MSH in which histidine, phenylalanine, arginine and tryptophan were present as D-isomers gave a very low melanocyte-stim-

ulating activity[33].

As a working hypothesis for further investigations into the specific structural demands of the amino acid residue at position 9 in natural ACTH and α-MSH, we have supposed that the known electron donor properties of the indolyl side-chain in tryptophan[34] might be related to the apparent, but as yet undefined, functional importance of this amino acid in these hormonal factors. Replacement of tryptophan by an amino acid having comparable donor properties seemed a useful method to test this hypothesis. Schwyzer *et al.* found that the donor properties of tryptophan are comparable with those of DL-pentamethylphenylalanine[35]. Therefore, we decided to synthesize this artificial amino acid, to resolve it into its antipodes and to incorporate the L-enantiomer into ACTH-(1-24). For comparison, tryptophan was also replaced by an amino acid having no, or almost no, charge-transfer properties, *viz.* phenylalanine.

Since the ACTH-(1-10) sequence is the same as in α-MSH, we also decided to synthesized α-MSH analogues with tryptophan[9] replaced by L-pentamethylphenylalanine and L-phenylalanine.

I. 3 STRATEGY, COUPLING METHODS AND PROTECTIVE GROUPS EMPLOYED IN THE SYNTHESIS OF ACTH AND α-MSH PEPTIDES

The principle of carboxyl activation of an amino-protected amino acid or peptide followed by reaction of the activated derivative with a carboxyl-protected amino acid or peptide, forms the basis of almost all peptide syntheses. In order to obtain an homogeneous product, all other reactive groups than those involved in the formation of the new peptide bond have to be blocked temporarily in both reacting components[36].
Surveys of protecting groups and peptide-forming agents suitable for the synthesis of corticotrophic peptides have been given previously[2,37] and provided a useful basis for our synthetic investigation.

The most widely employed amino-protecting groups are the benzyloxycarbonyl group, introduced in 1932 by Bergmann and Zervas[38], and the *tert.* butyloxycarbonyl group, first introduced by Carpino[39] as a general protecting group for nitrogen in organic compounds. This was later adapted for use in peptide synthesis by McKay and Albertson[40] and, independently, by Anderson and McGregor[41]. Since the benzyloxycarbonyl group is labile to catalytic hydrogenolysis and the *tert.* butyloxycarbonyl group resists hydrogenolysis (labile to acids), these groups can be used together when amino groups are present in the side-chains. A newly developed acid-stable, alkaline-labile amino-

protective group, the methylsulphonylethyloxycarbonyl (Msc-) group[42] has also been used in our syntheses of ACTH peptides.

The most general method for reversible blocking of carboxylic groups is esterification. The alkali-stable *tert.* butyl ester[43] (generally used for the side-chains of glutamic and aspartic acid) and the alkali-labile methyl (or ethyl) ester[44] have found wide employment in the synthesis of corticotrophic peptides.

As to the sequence in which the various peptide bonds have to be formed, it is generally accepted that in the syntheses of longer peptides, condensation of peptide fragments is preferable to stepwise synthesis because the problem of purification becomes more severe in the latter.

Because the danger of racemization in coupling steps does not exist when the carboxyl activated component has a glycine residue as the *C*-terminal end, syntheses of corticotrophic peptides always employ coupling of an *N*-terminal decapeptide with the desired *C*-terminal peptide as the final step. Both the decapeptide and the relevant *C*-terminal fragments can be built up from smaller fragments using procedures which minimize the danger of racemization. Such methods include azide coupling, which was originally introduced by Curtius[45] and modified by Honzl and Rudinger[46], and the carbodiimide method[47] with the addition of *N*-hydroxy-

succinimide[48] or 1-hydroxybenzotriazole[49]. Syntheses of these smaller fragments can then be done stepwise as well as by fragment condensation *via* active esters (*i.e.* *p*-nitrophenyl ester[50]) or by employing the mixed anhydride method[51]. Besides these methods we have also employed the triphenyl phosphite method of Mitin and Glinskaya[52] in the synthesis of small fragments.

I. 4 SYNOPSIS OF THE THESIS

In chapter II the synthesis and resolution of pentamethylphenylalanine is described.

Chapter III deals with the synthesis of two tetracosapeptides with pentamethylphenylalanine substituted for tryptophan[9], *viz.* [Pmp[9]]- and [D-Ser[1], Pmp[9]]-β-corticotrophin-(1-24)-tetracosapeptides. The latter analogue was synthesized as it has been shown that the presence of the D-isomer at the *N*-terminal position slows down enzymatic degradation of corticotrophins and consequently can enhance biological potencies in the usual test systems[53].

The synthesis of [Phe[9]]- and [D-Ser[1], Phe[9]]-β-corticotrophin-(1-24)-tetracosapeptides is described in chapter IV.

In chapter V the synthesis of [Pmp[9]]- and [Phe[9]]-α-MSH is described.

Chapter VI deals with the electron donor properties of several tryptophan containing peptides and the corresponding pentamethylphenylalanine and phenylalanine compounds.

In chapter VII the biological properties of the four ACTH- and two α-MSH analogues are summarized.

The synthesis of the ACTH-(7-10)-tetrapeptide with arginine^{8} replaced by norarginine is described in chapter VIII, while the synthesis and biological activity of [Nar^{8}]-β-corticotrophin-(1-24)-tetracosapeptide and of [Nar^{8}, Lys^{17,18}]-β-corticotrophin-(1-18)-octadecapeptide amide are given in chapter IX. The octadecapeptide amide analogue was synthesized for comparison with the previously investigated homoarginine^{8}-peptide which had the same number of amino acid residues.

REFERENCES

1. Riniker, B., Sieber, P., Rittel, W. and Zuber, H. (1972) *Nature (London), New Biol.* 235, 114-115.
2. For reviews see:
   Hofmann, K. (1962) *Ann. Rev. Biochem.* 31, 213-246.
   Hofmann, K. and Katsoyannis, P.G. (1963) in The Proteins (Neurath, H., ed.) vol. I, pp. 53-188, Academic Press, New York.
   Schröder, E. and Lübke, K. (1966) The Peptides, vol. 2, Synthesis, Occurrence, and Action of Biologically Active Polypeptides, pp. 194-251, Academic Press, New York,
   Li, C.H. and Pedersen, K.O. (1967) in The Adrenal Cortex (Eisenstein, A.B., ed.) pp. 185-201, Little, Brown and Co., Boston.
   Ramachandran, J. and Li. C.H. (1967) in Advances in Enzymology (Nord, F.F., ed.) vol. 29, pp. 391-477, Interscience Publishers, New York.
   Ramachandran, J. (1973) in Hormonal Proteins and Peptides (Li, C.H., ed.) vol. II, pp. 1-28, Academic Press, New York.
   Hofmann, K. manuscript in preparation.
3. Schwyzer, R. (1964) *Ann. Rev. Biochem.* 33, 259-286.
4. Ramachandran, J., Chung, D. and Li, C.H. (1965) *J. Amer. Chem. Soc.* 87, 2696-2708.
5. Hofmann, K., Wingender, W. and Finn, F.M. (1970) *Proc. Natl. Acad. Sci.* USA 67, 829-836.
6. Finn, F.M., Widnell, C.C. and Hofmann, K. (1972) *J. Biol. Chem.* 18, 5695-5702.
7. Schwyzer, R. (1973) in Peptides 1972, Proceedings of the 12th European Peptide Symposium (Hanson, H. and Jakubke, H.-D., eds.) pp. 424-436, North-Holland Publishing Co., Amsterdam, and references therein.

8. Schwyzer, R., Schiller, P., Fauchère, J.-L., Karlaganis, G. and Pelican, G.-M. (1972) in Structure-Activity Relationships of Protein and Polypeptide Hormones, Proceedings of the 2nd International Symposium, Liège, 1971 (Margoulies, M. and Greenwood, F.C., eds.) pp. 167-175, Excerpta Medica, Amsterdam, and references therein.
9. Seelig, S., Sayers, G., Schwyzer, R. and Schiller, P. (1971) *FEBS Lett.* 19, 232-234, and references therein.
10. Smith, P.E. (1916) *Science* 44, 280-282.
11. Allen, B.M. (1916) *Science* 44, 755-757.
12. Li, C.H., Schnabel, E., Chung, D. and Lo, T.-B. (1961) *Nature* 189, 143.
13. Otsuka, H. and Inouye, K. (1964) *Bull. Chem. Soc. Japan* 37, 1465-1471.
14. Desaulles, P.A., Barthe, P., Schär, B. and Staehelin, M. (1966) *Acta Endocrinol.* 51, 609-618.
15. Steelman, S.L. and Guillemin, R. (1959) *Proc. Soc. Exp. Biol. Med.* 101, 600-601.
16. Hofmann, K. manuscript in preparation.
17. Tesser, G.I. and Schwyzer, R. (1966) *Helv. Chim. Acta* 49, 1013-1022.
18. Tesser, G.I. and Rittel, W. (1969) *Rec. Trav. Chim.* 88, 553-561.
19. Tesser, G.I. and Buis, J.T. (1971) *Rec. Trav. Chim.* 90, 444-457.
20. Chung, D. and Li, C.H. (1967) *J. Amer. Chem. Soc.* 89, 4208-4213.
21. Tesser, G.I., Pleumekers, A.W.J., Bassie, W. and Belvert-Geers, I.C. (1973) *Rec. Trav. Chim.* 92, 1210-1222.
22. Tesser, G.I., Maier, R., Schenkel-Hulliger, L., Barthe, P.L., Kamber, B. and Rittel, W. (1973) *Acta Endocrinol.* 74, 56-66.

23. Dedman, M.L., Farmer, T.H. and Morris, C.J.O.R. (1957) *Biochem. J.* 66, 166-177.
24. Hofmann, K., Andreatta, R., Bohn, H. and Moroder, L. (1970) *J. Med. Chem.* 13, 339-345.
25. Fujino, M., Hatanaka, C., Nishimura, O., Nakayama, R., Shitaka, M. and Fujiwara, R. (1971) in Proceedings of the 8th Symposium on Peptide Chemistry, Osaka 1970 (Kaneko, T., ed.) pp. 101-105, Protein Research Foundation, Osaka;
Chem. Pharm. Bull. (1971) 19, 1075-1077.
26. Ramachandran, J. and Lee, V. (1970) *Biochem. Biophys. Res. Commun.* 38, 507-512.
27. Ramachandran, J. (1970) *Biochem. Biophys. Res. Commun.* 41, 353-357.
28. Moyle, W.R., Kong, Y.C. and Ramachandran, J. (1973) *J. Biol. Chem.* 248, 2409-2417.
29. Sayers, G., Swallow, R.L. and Giordano, N.D. (1971) *Endocrinol.* 88, 1063-1068.
30. Seelig, S., Kumar, S. and Sayers, G. (1972) *Proc. Soc. Exp. Biol. Med.* 139, 1217-1219.
31. Yajima, H. and Kubo, K. (1965) *Chem. Pharm. Bull.* 13, 759-764.
32. Koida, M., Hano, K. and Iso, T. (1966) *Japan. J. Pharmacol.* 16, 243-249.
33. Yajima, H. and Kawasaki, K. (1968) *Chem. Pharm. Bull.* 16, 1387-1390.
34. Pullman, B. and Pullman, A. (1958) *Proc. Natl. Acad. Sci.* USA 44, 1197-1202.
Szent-Györgyi, A., Isenberg, J. and McLaughlin J. (1961) *Proc. Natl. Acad. Sci.* USA 47, 1089-1093.
Cilento, G. and Tedeschi, P. (1961) *J. Biol. Chem.* 236, 907-910.
Shifrin, S. (1964) *Biochemistry* 3, 829-833;
Biochim. Biophys. Acta 81, 205-213.

35. Carrión, J.P., Donzel, B., Deranleau, D.A., Esko, K., Moser, P. and Schwyzer, R. (1967) in Peptides 1966, Proceedings of the 8th European Peptide Symposium (Beyerman, H.C., Van de Linde, A. and Maassen van den Brink, W., eds.) pp. 177-188, North-Holland Publishing Co., Amsterdam;
Helv. Chim. Acta (1968) 51, 459-481.

36. Jakubke, H.-D. and Jeschkeit, H. (1973) Aminosäure-Peptide-Proteine, Eine Einführung, Akademie-Verlag, Berlin.
Amino Acids, Peptides and Related Compounds (Hey, D.H. and John, D.I., eds.) Organic Chemistry Series One, vol. 6, 1973 (MTP International Review of Science), Butterworths, London.
Law, H.D. (1970) The Organic Chemistry of Peptides, Wiley-Interscience, London.
Schröder, E. and Lübke, K. (1965) The Peptides, vol. 1, Methods of Peptide Synthesis, Academic Press, New York.
Greenstein, J.P. and Winitz, M. (1961) Chemistry of the Amino Acids, vol. 2, pp. 763-1295, J. Wiley & Sons, Inc., New York.
Amino Acids, Peptides and Proteins (Young, G.T., ed.) vol. 1-4, Specialist Periodical Reports 1968-1971, The Chemical Society, Burlington House, London.
Klausner, Y.S. and Bodanszky, M. (1972) *Synthesis*, 453-463.
Kapoor, R. (1970) *J. Pharm. Sci.* 59, 1-26.

37. Schwyzer, R. and Kappeler, H. (1963) *Helv. Chim. Acta* 46, 1550-1572.
Schwyzer, R. and Sieber, P. (1965) *Helv. Chim. Acta* 49, 134-158.

38. Bergmann, M. and Zervas, L. (1932) *Chem. Ber.* 65, 1192-1201.

39. Carpino, L.A. (1957) *J. Amer. Chem. Soc.* 79, 98-101.
40. McKay, F.C. and Albertson, N.F. (1957) *J. Amer. Chem. Soc.* 79, 4686-4690.
41. Anderson, G.W. and McGregor, A.C. (1957) *J. Amer. Chem. Soc.* 79, 6180-6183.
42. Tesser, G.I. and Balvert-Geers, I.C. in preparation.
43. Anderson, G.W. and Callahan, F.M. (1960) *J. Amer. Chem. Soc.* 82, 3359-3363.
44. Curtius, T. (1883) *Ber. Deutschen Chem. Ges.* 16, 753-757.
Fischer, E. (1901) *Ber. Deutschen Chem. Ges.* 34, 433-454.
Brenner, M. and Huber, W. (1953) *Helv. Chim. Acta* 36, 1109-1115.
45. Curtius, T. (1902) *Ber. Deutschen Chem. Ges.* 35, 3226-3228.
46. Honzl, J. and Rudinger, J. (1961) *Coll. Czech. Chem. Commun.* 26, 2333-2344.
47. Sheehan, J.C. and Hess, G.P. (1955) *J. Amer. Chem. Soc.* 77, 1067-1068.
48. Wünsch, E. and Drees, F. (1966) *Chem. Ber.* 99, 110-120.
Weygand, F., Hoffmann, D. and Wünsch, E. (1966) *Z. Naturforschg.* 21b, 426-428.
49. König, W. and Geiger, R. (1971) in Peptides 1969, Proceedings of the 10th European Peptide Symposium (Scoffone, E., ed.) pp. 17-22, North-Holland Publishing Co., Amsterdam;
Chem. Ber. (1970) 103, 788-798.
50. Bodanszky, M. (1955) *Nature* 175, 685.
51. Anderson, G.W., Zimmerman, J.E. and Callahan, F.M. (1967) *J. Amer. Chem. Soc.* 89, 5012-5017.
52. Mitin, Y.V. and Glinskaya, O.V. (1969) *Tetrahedron Letters* 5267-5270.

53. Kappeler, H., Riniker, B., Rittel, W., Desaulles, P.A., Maier, R., Schär, B. and Staehelin, M. (1967) in Peptides 1966, Proceedings of the 8th European Peptide Symposium (Beyerman, H.C., Van de Linde, A. and Maassen van den Brink, W., eds.) pp. 214-220, North-Holland Publishing Co., Amsterdam.

CHAPTER II

SYNTHESIS AND RESOLUTION OF DL-PENTAMETHYLPHENYLALANINE

II. 1 INTRODUCTION

As already mentioned in chapter I, our intention was to synthesize a new amino acid with electron donor properties and to replace tryptophan in a natural peptide by this amino acid in order to investigate the possible role of tryptophan as an electron donor in biologically active peptides, particularly in the adrenocorticotrophic hormone.

Carrión *et al.*[1,2] have already pointed out that the side-chain of DL-pentamethylphenylalanine possesses charge donor properties comparable with those of the indole side-chain of tryptophan. Therefore, we decided to synthesize this amino acid, to resolve it into its enantiomers and to insert the L-isomer into ACTH.

From the host of methods used for the preparation of amino acids[3], the most convenient one in this case seemed to be the reaction between the appropriate alkyl chloride and diethyl acetamidomalonate[4]. The resulting intermediate can lead either to the free amino acid by treatment with hydrogen bromide, or to the *N*-acetyl derivative by hydrolysis of the ethyl ester functions with aqueous sodium hydroxide and decarboxylation.

Enzymatic resolution of acetyl derivatives of amino acids is a general method. Chemical resolution can also be attempted with such compounds but then an energic hydrolysis must be employed to afford the free amino acid.

Finally, the assignment of the configuration of new amino acids can be performed with the aid of optical rotatory dispersion measurements[5].

II. 2 SYNTHESIS OF N-ACETYL-DL-PENTAMETHYLPHENYL-ALANINE

For the application of the procedure of Albertson and Archer[4] in the synthesis of the amino acid derivative, diethyl acetamidomalonate and pentamethylbenzyl chloride were necessary as the starting compounds; the chloride in its turn was synthesized from mesitylene (scheme II, 1).

In 1934 Von Braun and Nelles[6] introduced one or two chloromethyl groups into aromatic hydrocarbons with hydrogen chloride and formaldehyde. Nauta and Dienske[7] chloromethylated mesitylene (I) under varying conditions. They obtained about 15% of the dichloromethyl compound (II) using equimolar amounts of formaldehyde and mesitylene. Using two equivalents of formaldehyde, the yield increased up to 72%. Shacklett and Smith[8], using Von Braun's method, found that a second addition of formaldehyde and subsequent heating was unnecessary and succeeded in obtaining some 80% bis(chloromethyl)-mesitylene.

Several procedures have been described for the reduction of chloromethylated mesitylene. Vavon and Bolle[9] claimed that the chloromethyl group can be transformed into a methyl group without hydrogenation of the ring by limited hydrogenation in the presence of platina.

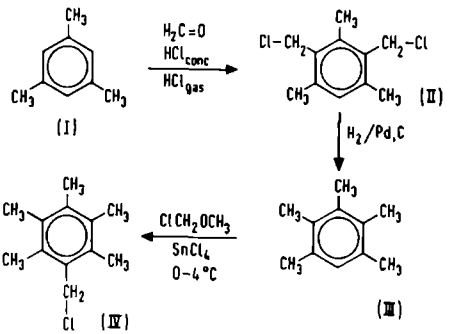
Aitken *et al.*[10] have tried to reduce bis(chloromethyl)mesitylene with hydrogen and palladised asbestos in acetone; this method appeared to be tedious, however, and gave incompletely reduced products. Reduction of the chloromethyl group with zinc and aqueous sodium hydroxide solution in benzene by Von Braun and Nelles[6] resulted in some 70% of pentamethylbenzene (III). Zinc and aqueous sodium hydroxide gave mainly non-crystalline undistillable products however, and the same is true on reduction with a nickel-aluminum alloy and aqueous sodium hydroxide at 90°C. The reaction was most readily accomplished by stirring the product in toluene with zinc and aqueous sodium hydroxide. The yield of pentamethylbenzene was then higher than with benzene as the solvent.

Finally, Shacklett and Smith[8] obtained poor yields in the hydrolysis of the Grignard reagents of chloromethylated aromatics, but they obtained the reduction product in nearly quantitative yield when the halide was treated with lithium hydride or lithium aluminum hydride in tetrahydrofuran solution.

We obtained a nearly quantitative yield by simple hydrogenation of the bis(chloromethyl) com-

pound (II) in acetic acid with palladium on charcoal which consequently proved to be the method of choice.

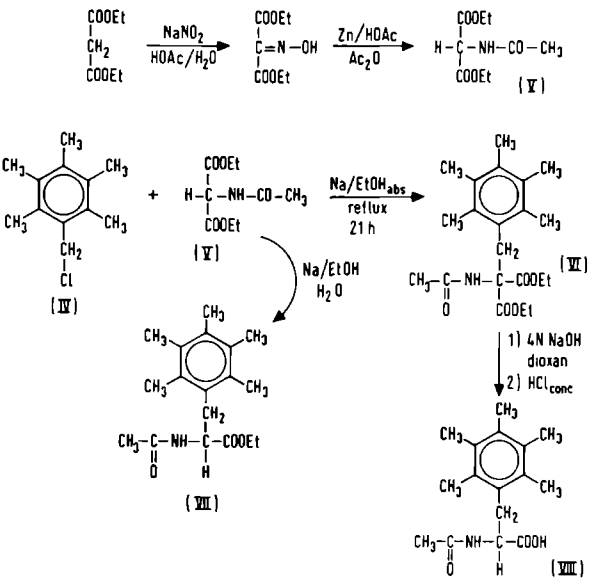
The synthesis of pentamethylbenzyl chloride (IV) from pentamethylbenzene has been carried out by treatment with chloromethyl methyl ether[11] and acetic acid at 80°C for about 17 hours[12,13]. According to Pepper[11] a much smoother reaction takes place with chloromethyl methyl ether and tin tetrachloride at low temperature.



Scheme II, 1

The other starting compound, diethyl acetamidomalonate (V), was prepared according to the method of Zambito and Howe[14]. Coupling of this malonate with pentamethylbenzyl chloride was performed in absolute ethanol with sodium ethylate[1,4]. Traces of water do not impair the alkylation of the malonate, but the product isolated is N-acetyl-DL-pentamethylphenylalanine ethyl ester (VII) rather than the malonate (VI).

By alkaline hydrolysis and subsequent decarboxylation of the diethyl 2-pentamethylbenzyl-2-acetamido malonate (VI), the racemic pentamethylphenylalanine derivative (VIII) was obtained.



Scheme II, 2

II. 3 RESOLUTION AND ASSIGNMENT OF CONFIGURATION

According to Greenstein and Winitz[15] resolution can be achieved:

a) by selective crystallization
b) biologically, by means of specific enzymes, as for instance carboxypeptidase, renal and mould acylase (carboxypeptidase is to be preferred when aromatic amino acids are involved)
c) by chemical means

Because method a) can only be used if one of the isomers is available we tried the enzymatic method b) as applied in the resolution of phenylalanine. L-phenylalanine is obtained in fairly good yield by digestion of chloroacetyl-DL-phenylalanine

or the acetyl compound with hog renal acylase I or carboxypeptidase, respectively[16].

In our case the turnover rate of the hydrolysis with carboxypeptidase A[17] proved impracticably slow (0.5 µmol/mg/h) for a usable resolution. Enzymatic hydrolysis has been used, later on, to corroborate the assigned configuration.

Chemical resolution of acids has mostly been carried out with alkaloids such as brucine, ephedrine, quinine and cinchonine, or other optically active bases such as α-phenylethylamine, dexamphetamine and α-fenchylamine. Overby and Ingersoll[18] unsuccessfully attempted to resolve *N*-acetyl-DL-phenylalanine with α-phenylethylamine, ephedrine, brucine, cinchonine and quinine. A resolution giving both forms in the pure state was easily effected, however, with optically active α-fenchylamines. With the (-)amine mainly the salt of the acetyl-D-amino acid precipitated and was readily obtained in pure form in 80-93% yield. The salt of the acetyl-L-amino acid proved to be very soluble and remained in the mother liquor. On decomposition of the separated salts by treatment with alkali, the amine was nearly quantitatively recovered and pure acetyl-D-amino acid and partially resolved acetyl-L-amino acid were obtained. Pure acetyl-L-phenylalanine was then obtained from the partially resolved material by one or two crystallizations from acetone, in which the accompanying DL-form is more soluble. In this way complete resolution was effected with only one form of the resolving agent.

For the resolution of our acetyl-DL-pentamethylphenylalanine several optically active bases such as (-)ephedrine, (+)dexamphetamine, quinine and brucine were tried. It appeared that only the brucine salts of the acetylated antipodes differed sufficiently in solubility to obtain good resolution.

A favourable feature of the procedure was the independent crystallizability of both diastereomeric salts from different solvent systems once they had been roughly separated. So ethyl acetate/propanol-2 preferentially dissolves the salt containing the dextrorotating acid, and methanol the other. Methanol is also an excellent solvent for the mixture of both salts. Thus, the fundamental problem in chemical resolutions concerning the stereochemical purity of the "second, better soluble" fraction did not arise in this case.

Decomposition of both diastereomeric salts was carried out with an excess of hydrochloric acid in propanol-2. When a primary alcohol was used, partial esterification occurred rapidly. The antipodes were isolated in nearly theoretical yield, and their absolute specific rotations were equal within the limits of experimental error.

Hydrolysis of the acetylated antipodes was performed by heating their solutions in acetic acid with concentrated aqueous hydrochloric acid under reflux. In both cases the product crystallized on cooling in the form of its monohydrochloride monohydrate. (On drying in a high vacuum at 80°C over P_2O_5, efflorescence was observed and an anhydrous hydrochloride was left. In moist air the hydrate

reconstituted itself.) Treatment of its solution in acetic acid with ammonium acetate gave the free amino acid.

As the deacetylation procedure was rather drastic, the risk of partial racemization at this step is not merely theoretical. Perceptible racemization could be ruled out by reacetylation of a sample: the rotation of the product appeared to be unaltered.

The resolution was performed twice, with different batches of brucine. The results supported each other.

Assignment of the configuration was performed by rotatory dispersion measurements[5]. Positive Cotton effects were recorded for dextrorotating *N*-acetyl-pentamethylphenylalanine as well as for *N*-acetyl-L-phenylalanine and L-phenylalanine. The (+)-enantiomer was also susceptible to enzymatic attack: digestion with carboxypeptidase A, although very slow, resulted in precipitation of the free amino acid after one day of incubation. The optical rotatory dispersion curve of the precipitated amino acid also revealed a positive Cotton effect. The levorotating enantiomer was not digestible with carboxypeptidase A.

From these observations we attribute L-configuration to (+)-acetyl-pentamethylphenylalanine and D-configuration to its enantiomer. As deacetylation did not alter the sign of the rotation, dextrorotatory pentamethylphenylalanine has to be looked upon as belonging to the class of amino acids having "natural" asymmetry.

II. 4 EXPERIMENTAL SECTION

For details concerning abbreviations, thin-layer chromatography and performance of measurements, see appendices.

Scheme II, 1.

Bis(chloromethyl)mesitylene (II)

1 Mole of mesitylene (120 g = 139.5 ml), 2.65 moles of formaldehyde (as a 37% solution = 240 g = 220.5 ml) and 7.36 moles (480 g = 610.5 ml) of a concentrated HCl solution were mixed together in a three-necked round bottom flask, equipped with a stirrer, a condenser and an inlet tube for gaseous HCl. While stirring at 65°C HCl was introduced and a precipitate separated. After 5 hours, water was added and the precipitate was filtered, thoroughly washed with water and dried. Upon recrystallization from acetic acid 78% of II was obtained; it still contained a small quantity of tri(chloromethyl)mesitylene.
M.p. 103-105°C. Lit.[7] 105°C, lit.[19] 105-106°C. N.M.R. (CCl_4): δ 7.15 (s, 1H, ϕ-H), 4.90 (s, 4H, CH_2Cl), 2.76 (s, 3H, p-CH_3), 2.68 (s, 6H, o-CH_3).
Analysis:

$C_{11}H_{14}Cl_2$	Calcd.:	% C 60.85	% H 6.50	% Cl 32.65
(217.14)	Found :	% C 61.0	% H 6.3	% Cl 33.9

Pentamethylbenzene (III)

Catalytic hydrogenation of II with palladium on charcoal was performed in acetic acid. On concentration of the filtered solution some hexamethylbenzene crystallized. Addition of water and cooling followed by filtration and drying gave 94% of III.
M.p. 52-53°C. Lit.[6] 51°C. N.M.R. (CCl_4): δ 6.96 (s, 1H, ϕ-H), 2.45 (m, 15H, ring CH_3).

Determination of the chlorine content indicated that about 0.9% Cl remained.

Pentamethylbenzyl chloride (IV)

72.1 g of pentamethylbenzene (0.49 moles) were dissolved in 200 ml of chloroform (p.a.) and the solution was cooled in an ice bath. A solution of 73.3 ml of chloromethyl methyl ether (0.96 moles) and 12 ml of tin tetrachloride (0.13 moles) (both freshly distilled) was added stepwise. The solution was stirred for 16 hours at 0-4°C. After addition of water, the chloroform layer was separated, washed with water and saturated NaCl solution, dried over Na_2SO_4 and evaporated. The residue was recrystallized from 90% acetic acid yielding 85% of pure IV with m.p. 82-84°C. Lit.[12] 80°C, [19,20] 80-82°C, [2] 80-82.5°C, [8] 82-84°C, [21] 99°C and [13] 161-165°C. N.M.R. ($CDCl_3$): δ 4.64 (s, 2H, CH_2Cl), 2.29 (s, 6H, *o*-CH_3), 2.16 (s, 9H, *m*- and *p*-CH_3). Lit.[20]: N.M.R. ($CDCl_3$): δ 4.74, 2.37 and 2.25.

Analysis:

$C_{12}H_{17}Cl$	Calcd.:	% C 73.27	% H 8.71	% Cl 18.02
(196.72)	Found :	% C 73.5	% H 8.9	% Cl 18.1

Scheme II, 2.

Diethyl acetamidomalonate (V)

The malonate was prepared according to the method of Zambito and Howe. Recrystallization from water (the hot solution must be stirred vigorously upon cooling) yielded 72.5% of V, m.p. 94-96°C. Lit.[14] 95-97°C. TS: Rf = 0.55 (A) (I_2). N.M.R. (DMSO-d_6): δ 8.95 (d, 1H, NH, J_{NH-CH} = 7 cps), 5.29 (d, 1H, CH), 4.38 (q, 4H, $O\underline{CH_2}$-CH_3, J = 7 cps), 2.11 (s, 3H, CH_3-CO-), 1.39 (t, 6H, OCH_2-$\underline{CH_3}$).

Analysis:

$C_9H_{15}NO_5$	Calcd.:	% C 49.76	% H 6.96	% N 6.45
(217.22)	Found :	% C 49.7	% H 7.1	% N 6.4

Diethyl(2-pentamethylbenzyl-2-acetamido)malonate (VI)

62.5 g of pentamethylbenzyl chloride (317.2 mmoles) and 84 g of V (382.1 mmoles) were added to a clear and colourless solution of 8.0 g of sodium in about 1600 ml of dried and freshly distilled ethanol. The solution was refluxed for 20 hours and then filtered and concentrated. On addition of some ethyl acetate and petroleum ether about 80% of VI precipitated. The product was purified by crystallization from 70% acetic acid, giving large rectangular plates. Yield: 70%. M.p. 133-135°C. Lit.[2] (99°C) 112-114°C (125°C). TS: Rf = 0.60 (A) (R-H). N.M.R. (CCl_4): δ 6.61 (s, 1H, NH), 4.35 (several quartets (4?) due to hindered rotation, 4H, $O\underline{CH_2}$-CH_3, J = 7 cps), 4.00 (s, 2H, φ-CH-), 2.39 (m, 15H, ring CH_3), 2.12 (s, 3H, CH_3-CO-), 1.48 (t, 6H, OCH_2-$\underline{CH_3}$).

Analysis:

$C_{21}H_{31}NO_5$	Calcd.:	% C 66.82	% H 8.28	% N 3.71
(377.48)	Found :	% C 66.7	% H 8.3	% N 3.7

N-Acetyl-DL-pentamethylphenylalanine ethyl ester (VII)

When the reaction mixture in the preparation of VI contained some water, originating either from the ethanol or the malonate, a by-product separated on addition of ethyl acetate. M.p. 142-143°C. TS: Rf = 0.51 (A) (R-H). N.M.R. (CCl_4): δ 6.94 (d, 1H, NH, J_{NH-CH} = 8 cps), 5.86 (m, 1H, $\underline{CH}$-CH_2, J_{CH-CH_2} = 8 cps), 4.21 (q, 2H, $O\underline{CH_2}$-CH_3, $J_{OCH_2-CH_3}$ = 8 cps), 3.36 (d, 2H, φ-CH_2-), 2.48 (s, 6H, *o*-CH_3), 2.44 (s, 9H, *m*- and *p*-CH_3), 2.19 (s, 3H, CH_3-CO-), 1.28 (t, 3H, OCH_2-$\underline{CH_3}$).

Analysis:

$C_{18}H_{27}NO_3$	Calcd.:	% C 70.79	% H 8.91	% N 4.59
(305.42)	Found :	% C 70.6	% H 8.9	% N 4.5

When *N*-acetyl-DL-pentamethylphenylalanine (VIII) was dissolved in benzene/ethanol and refluxed for a few hours

with some *p*-toluenesulphonic acid, the same compound was produced, as judged from its melting point, Rf value and N.M.R. pattern.

N-Acetyl-DL-pentamethylphenylalanine (VIII)

By alkaline hydrolysis and decarboxylation of the foregoing compound, the racemic pentamethylphenylalanine derivative was obtained. Therefore, diethyl(2-pentamethylbenzyl-2-acetamido)malonate was dissolved in a mixture of dioxan and water (2:1). A solution of 4 N sodium hydroxide (about 20% excess) was added, and the solution refluxed for about 18 hours. Dioxan was removed under reduced pressure and the resulting solution extracted with ethyl acetate to remove some 5% of unreacted starting material which was recycled. The aqueous layer was poured into an excess of 6 N hydrochloric acid with stirring, then cooled and filtered. After recrystallization from 90% acetic acid, about 96% of a chromatographically pure product was obtained, m.p. 247-248°C, TS: Rf = 0.67 (B), = 0.81 (C) (R-H).

Analysis:

$C_{16}H_{23}NO_3$	Calcd.:	% C 69.29	% H 8.36	% N 5.05
(277.36)	Found :	% C 68.9	% H 8.5	% N 5.0

Resolution of N-acetyl-DL-pentamethylphenylalanine

Because of the limited solubility in the solvent system used for the resolution, 65.0 g (234.3 mmoles) of *N*-acetyl-DL-pentamethylphenylalanine and 92.3 g (234.3 mmoles) of brucine were dissolved in 650 ml of warm methanol. The solvent was removed by evaporation, and evaporation was repeated after the addition of some propanol-2 to eliminate residual methanol. The residue containing the diastereomeric brucine salts was dissolved in 300 ml of warm propanol-2, the same volume of ethyl acetate was added and the solution stored in a refrigerator for 7-8 days. After filtration and washing of the precipitate (A) with ethyl acetate, the com-

bined filtrates were evaporated to dryness, the residue taken up in ethyl acetate and the solvent evaporated again. To remove the last traces of propanol-2 this procedure was repeated. The residue was dissolved in some 300 ml of warm ethyl acetate and the solution was cooled and kept at about $0^{o}C$ for 1 week. A small amount of precipitate (B) was filtered and the filtrate evaporated to dryness. Following dissolution of the residue in 300 ml of warm methanol immediate crystallization occurred upon cooling, giving fraction C.

Fractions A and C were recrystallized from ethyl acetate/propanol-2 (1:1) and methanol, respectively.

Fraction	Yield	M.p.	$[\alpha]_D^{24}$ (c = 1.0, MeOH)
A	69.2 g = 43.9%	216-218°C	-16.0°
B	2.5 g = 1.6%	130-240°C	- 2.4°
C	57.8 g = 36.7%	129-134°C	- 6.2°

N-Acetyl-L-pentamethylphenylalanine

54.84 g of the brucine salt C (80.3 mmoles) were dissolved in 200 ml of propanol-2. Fifty ml of 4 N hydrochloric acid (200 mmoles) were added with stirring followed by about 1300 ml of water. After cooling, the precipitate was filtered, washed with dilute hydrochloric acid and dried.

Yield: 21.05 g = 94.5% of a chromatographically pure product; TS: Rf = 0.80 (C) (R-H). M.p. 246-248°C (the crystals melted at about 222°C, but then suddenly gave long thin needles, which melted again at 246-248°C). N.M.R. (DMSO-d_6): δ 8.39 (d, 1H, NH, J_{NH-CH} = 8 cps), 4.48 (m, 1H, $\underline{CH}$-CH_2), 3.20 (d, 1H, $C_\beta H_A$, $J_{C_\alpha H-C_\beta H_A}$ = 7 cps), 3.28 (d, 1H, $C_\beta H_B$, $J_{C_\alpha H-C_\beta H_B}$ = 9 cps), 2.37 (s, 6H, *o*-CH_3), 2.33 (s, 9H, *m*- and *p*-CH_3), 2.00 (s, 3H, CH_3-CO-).
$[\alpha]_D^{21}$ = +15.8° (c = 1.0, MeOH); $[\alpha]_{578}$ = +16.7°, $[\alpha]_{546}$ = +19.3°, $[\alpha]_{436}$ = +38.0°, $[\alpha]_{365}$ = +74.2°.

Analysis:

$C_{16}H_{23}NO_3$	Calcd.:	% C 69.29	% H 8.36	% N 5.05
(277.36)	Found :	% C 69.2	% H 8.5	% N 5.0

N-Acetyl-D-pentamethylphenylalanine was obtained from its brucine salt (A) in a similar manner in 88% yield. M.p.: 242-245°C. (The crystals melted at about 218°C, giving long thin needles which melted again at 242-245°C.) N.M.R.: identical with that given for the L-compound. $[\alpha]_D^{21}$ = -16.0° (c = 0.98, MeOH); $[\alpha]_{578}$ = -16.5°, $[\alpha]_{546}$ = -19.4°, $[\alpha]_{436}$ = -38.1°, $[\alpha]_{365}$ = -74.4°.

Analysis: Found: % C 69.3 % H 8.5 % N 5.0

L-Pentamethylphenylalanine monohydrochloride monohydrate

The *N*-acetyl compound (20.03 g = 72.2 mmoles) was dissolved in 200 ml of concentrated aqueous hydrochloric acid and 200 ml of glacial acetic acid, and the solution was refluxed for 5 hours. When heating was discontinued crystallization started at once, and 20.37 g = 97.5% of L-H-Pmp-OH.HCl.H_2O* could be isolated. TS: Rf = 0.50 (D) (R-H). M.p.: 256.5-258°C.

$[\alpha]_D^{21}$ = +55.2° (c = 0.59, HOAc + 0.1 N HCl (4:1)); $[\alpha]_{578}$ = +57.2°, $[\alpha]_{546}$ = +65.8°, $[\alpha]_{436}$ = +120.9°, $[\alpha]_{365}$ = +211.8°.

Analysis:

$C_{14}H_{24}NO_3Cl$	Calcd.:	% C 58.02	% H 8.35	% N 4.83	% Cl 12.23
(289.80)	Found :	% C 57.8	% H 8.3	% N 4.7	% Cl 12.4

* Suggested three-letter symbol for L-pentamethylphenylalanine is Pmp.

The same procedure was used to obtain the D-isomer, m.p. 254.5-256°C. $[\alpha]_D^{21}$ = -54.9° (c = 0.64, HOAc + 0.1 N HCl (4:1)); $[\alpha]_{578}$ = -57.0°, $[\alpha]_{546}$ = -65.7°, $[\alpha]_{436}$ = -120.3°, $[\alpha]_{365}$ = -211.3°.

Analysis: Found : % C 57.9 % H 8.3 % N 4.7 % Cl 12.4

L-Pentamethylphenylalanine

The free amino acid was obtained from its hydrochloride upon dissolution in hot acetic acid (100%), addition of an excess of ammonium acetate, and dilution with water. Consistent analytical data were obtained after thorough drying (80°C in high vacuum for about 6 hours). M.p.: 242-245°C.

$[\alpha]_D^{21}$ = +65.7° (c = 0.46, HOAc + 0.1 N HCl (4:1)); $[\alpha]_{578}$ = +68.8°, $[\alpha]_{546}$ = +78.5°, $[\alpha]_{436}$ = +144.7°, $[\alpha]_{365}$ = +254.4°.

Analysis:

$C_{14}H_{21}NO_2$	Calcd.:	% C 71.46	% H 9.00	% N 5.95
(235.33)	Found :	% C 71.5	% H 9.1	% N 5.9

The same procedure was used to obtain the D-isomer, m.p.: 243-246°C. $[\alpha]_D^{21}$ = -65.4° (c = 0.47, HOAc + 0.1 N HCl (4:1)); $[\alpha]_{578}$ = -68.3°, $[\alpha]_{546}$ = -78.1°, $[\alpha]_{436}$ = -144.0°, $[\alpha]_{365}$ = -254.3°.

Analysis: Found : % C 71.2 % H 9.0 % N 5.8

From the free amino acid UV spectra were recorded. In HOAc - 0.1 N HCl (4:1, v/v):

λ_{max} = 271.5 nm (ε = 372.0) and 277.0 nm (ε = 327.5); in 0.1 N NaOH:

λ_{max} = 296.0 nm (ε = 381.4) and λ_{min} = 249.0 nm (ε = 202.9).

REFERENCES

1. Carrión, J.-P., Donzel, B., Deranleau, D.A., Esko, K., Moser, P. and Schwyzer, R. (1967) in Peptides 1966, Proceedings of the 8th European Peptide Symposium, (Beyerman, H.C., van de Linde, A. and Maassen van den Brink, W., eds.) pp. 177-188, North-Holland Publishing Co., Amsterdam.
2. Carrión, J.-P., Deranleau, D.A., Donzel, B., Esko, K., Moser, P. and Schwyzer, R. (1968) *Helv. Chim. Acta* 51, 459-481.
3. Nivard, R.J.F. and Tesser, G.I. (1965) in Comprehensive Biochemistry (Florkin, M. and Stotz, E.H., eds.) vol. 6, pp. 143-207, Elsevier Publishing Co., Amsterdam.
4. Albertson, N.F. and Archer, S. (1945) *J. Amer. Chem. Soc.* 67, 308-310.
5. Dirkx, I.P. and Sixma, F.L.J. (1964) *Rec. Trav. Chim.* 83, 522-534.
6. Von Braun, J. and Nelles, J. (1934) *Chem. Ber.* 67, 1094-1099.
7. Nauta, W.Th. and Dienske, J.W. (1936) *Rec. Trav. Chim.* 55, 1000-1006.
8. Shacklett, C.D. and Smith, H.A. (1951) *J. Amer. Chem. Soc.* 73, 766-768.
9. Vavon, G. and Bolle, J. (1937) *Comptes Rendus* 204, 1826-1828.
10. Aitken, R.R., Badger, G.M. and Cook, J.W. (1950) *J. Chem. Soc.*, 331-335.
11. Pepper, K.W., Paisley, H.M. and Young, M.A. (1953) *J. Chem. Soc.*, 4097-4105.
    Pashkov, A.B., Epshtein, Y.V., Vdovin, N.M., Dmitriev, K.V., Naumov, S.N., Safin, R.S., Voronkova, O.N., Ermilova, T.I., Murzaev, V.S. and Kotlyarova, V.F. (1972) U.S.S.R. 325, 828 (Cl. C 07 c); *C.A.* (1972) 76, 139927 v.

12. Humphlett, W.J. and Hauser, C.R. (1950) *J. Amer. Chem. Soc.* 72, 3289-3290.
13. Deno, N.C., Groves, P.T., Jaruzelski, J.J. and Lugash, M.N. (1960) *J. Amer. Chem. Soc.* 82, 4719-4723.
14. Zambito, A.J. and Howe, E.E. (1960) in Organic Syntheses (Newman, M.S., ed.) vol. 40, pp. 21-23, J. Wiley & Sons Inc., New York.
15. Greenstein, J.P. and Winitz, M. (1961) Chemistry of the Amino Acids, vol. 1, p. 175, J. Wiley & Sons Inc., New York.
16. Greenstein, J.P. and Winitz, M. (1961) Chemistry of the Amino Acids, vol. 3, pp. 2172-2174, J. Wiley & Sons Inc., New York, and references therein.
17. Greenstein, J.P. (1954) in Advances in Protein Chemistry (Anson, M.L., Bailey, K. and Edsall, J.T., eds.) vol. 9, pp. 121-202, Academic Press, New York.
18. Overby, L.R. and Ingersoll, A.W. (1951) *J. Amer. Chem. Soc.* 73, 3363-3366.
19. Rhoad, M.J. and Flory, P.J. (1950) *J. Amer. Chem. Soc.* 72, 2216-2219.
20. Wasserman, H.H., Mariano, P.S. and Keehn, P.M. (1971) *J. Org. Chem.* 36, 1765-1769.
21. Jacobsen, O. (1889) *Ber. Deutschen Chem. Ges.* 22, 1215-1224.

CHAPTER III

SYNTHESIS OF TWO ACTH-(1-24) ANALOGUES CONTAINING L-PENTAMETHYLPHENYLALANINE IN POSITION 9

III. 1 INTRODUCTION

A widely accepted strategy for the synthesis of corticotrophic peptides is that used by Schwyzer and coworkers in the synthesis of the natural ACTH itself[1]. Applied to the synthesis of corticotrophic tetracosapeptides the last step in the formation of the protected end-product is a carbodiimide mediated coupling generating the glycyl-lysyl bond between a desired *N*-terminal decapeptide and the appropriate *C*-terminal fragment.

In our case the desired variation from the natural sequence consisted of the replacement of tryptophan by pentamethylphenylalanine in the mentioned *N*-terminal part. Therefore, we started by synthesizing [Pmp^{9}]-β-corticotrophin-(7-10)-tetrapeptide. The peptide was prepared *via* intermediates bearing conventional protecting groups (including the nitro function for the protection of the arginine side-chain) (scheme III,2), and also along a newly adopted route in which the alkali-labile methylsulphonylethyloxycarbonyl (Msc) group[2] was used for amino protection and the arginine side-chain was left unprotected (scheme III, 1). It appeared that the latter reaction pathway greatly shortened the pro-

cedure and gave rise to a purer end-product. In the former scheme in particular the deprotection steps led to difficultly crystallizable intermediates of poor quality, and to unsatisfactory yields.

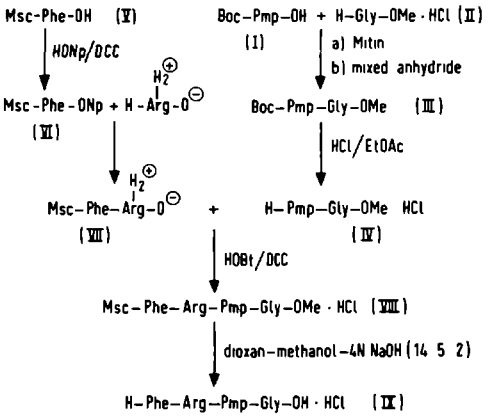
The tetrapeptide liberated from protection groups was then elongated at the *N*-terminal end by two successive azide couplings using first a di- and then a tetrapeptide (scheme III, 3). The latter was introduced in two different forms with *N*-terminal L- and D-Serine. Condensation of the zwitterionic decapeptide with the protected natural (11-24)-fragment[1] produced the desired tetracosapeptides which were converted into the free ACTH analogues by treatment with trifluoroacetic acid.

III. 2 METHOD OF SYNTHESIS

H-Phe-Arg-Pmp-Gly-OH

A. According to scheme III, 1.

The synthesis of this unprotected tetrapeptide was started by the introduction of the Boc group into L-pentamethylphenylalanine. With Schnabel's method[3] only a low yield was obtained due to the low solubility of Pmp which decreases in solutions of increasing ionic strengths; a precipitate was formed after 20-30% conversion (6 observations) and the reaction stopped. With a 15-20 fold excess of the acylating agent (Boc-N_3), however, precipitation could be avoided and excellent yields were obtained. We speculate that



Scheme III, 1

the organic phase of the reaction mixture is partly solubilized by micelles of the sodium salt of pentamethylphenylalanine, thus preventing its "salting-out" by a profound alteration in the solution structure.

Coupling of the protected amino acids I and II was carried out *via* Mitin's method[4] as well as *via* the mixed anhydride method of Anderson[5]. The methods gave similar results. Deprotection of the dipeptide III with hydrogen chloride in ethyl acetate yielded the hydrochloride IV as a crystalline compound.

Methylsulphonylethyloxycarbonyl-L-phenylalanine, a water-soluble compound, was condensed as its *p*-nitrophenyl ester with L-arginine (free base). The resulting dipeptide was crystallized from water, and then directly condensed with the hydrochloride IV according to König's method[6]. It gave the protected tetrapeptide ester VIII in approximately 95%

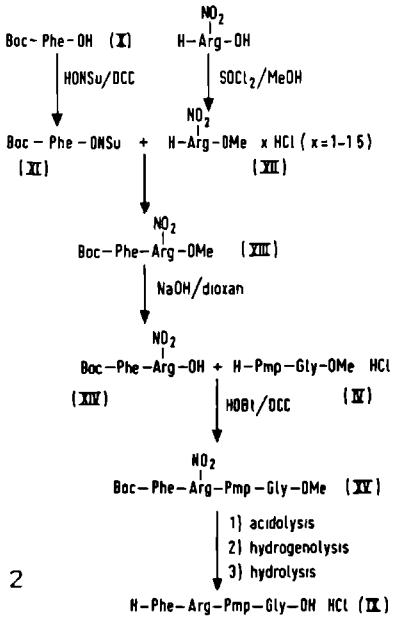
yield. The crude product was completely digestible with trypsin. Minor impurities in the crude product were largely removed by the "short-column" chromatographic procedure of Hunt and Rigby[7].

Deprotection of VIII with aqueous sodium hydroxide in dioxan appeared to be complete within a few minutes; when methanol was added (dioxan-methanol-4N sodium hydroxide solution = 14:5:1, 0.2 N in NaOH) the deprotection proved to be only a matter of seconds and a chromatographically pure product could be isolated.

B. According to scheme III, 2.

After introduction of the *t*-butyloxycarbonyl group into L-phenylalanine, the 1-succinimidyl ester (XI) was prepared according to the method of Anderson[8]. This activated ester was added to a solution containing the methyl ester of nitro-arginine after neutralization of the hydrogen chloride present in the ester. The resulting dipeptide ester (XIII) was hydrolyzed with a dioxan-aqueous sodium hydroxide solution, yielding some 90% of XIV. Coupling of XIV and IV with 1-hydroxybenzotriazole and dicyclohexylcarbodiimide[6] gave the fully protected tetrapeptide which appeared to be pure after recrystallization from ethanol.

Treatment with hydrogen chloride in ethyl acetate followed by hydrogenation in the presence of palladium on charcoal did not result in the

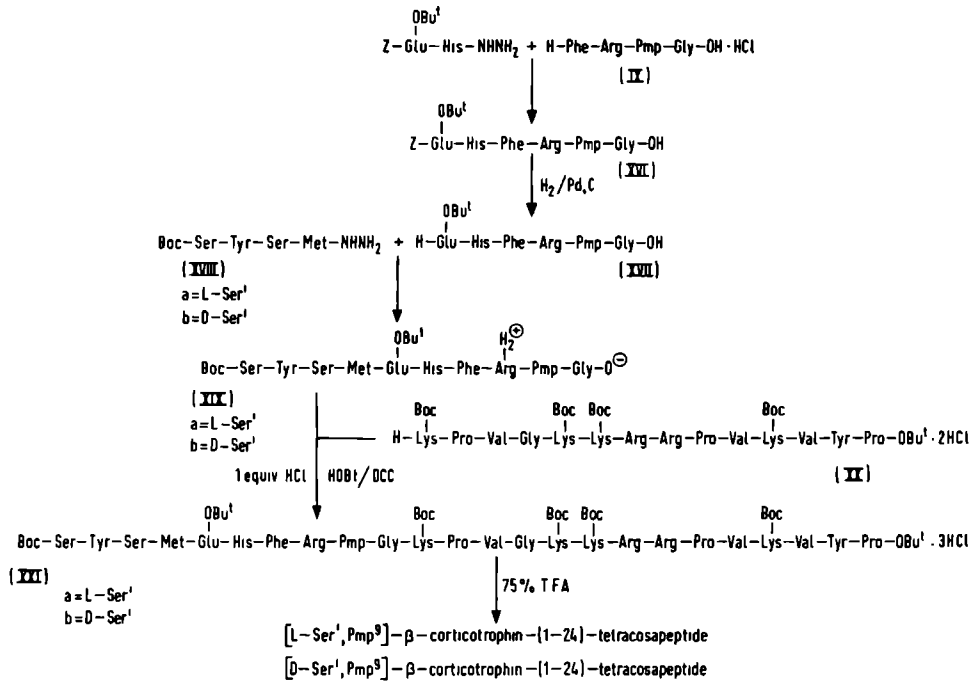


Scheme III, 2

pure deprotected ester. Several attempts at improvement of the procedure, in order to avoid the necessity of a time-consuming purification of the ester, were unsuccessful.

[Pmp^9]- and [D-Ser^1, Pmp^9]-β-corticotrophin-(1-24)-tetracosapeptides (scheme III, 3)

Acylation of the free tetrapeptide IX with Z-Glu(OBu^t)-His-N_3 was performed according to the *in situ* procedure of Honzl and Rudinger[9] using a 50% excess of the acylating azide. The resulting protected hexapeptide XVI was directly subjected to hydrogenolysis to give XVII. This intermediate was then similarly acylated with one of the azides derived from XVIII a or b. The ensuing decapeptide


OBu^t
Z-Glu-His-NHNH2 + H-Phe-Arg-Pmp-Gly-OH · HCl
(IX)
OBu^t
Z-Glu-His-Phe-Arg-Pmp-Gly-OH
(XVI)
H2/Pd,C
OBu^t
Boc-Ser-Tyr-Ser-Met-NHNH2 + H-Glu-His-Phe-Arg-Pmp-Gly-OH
(XVIII)
a=L-Ser^1
b=D-Ser^1
(XVII)
OBu^t
H2^⊕
Boc-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Pmp-Gly-O^⊖
(XIX)
a=L-Ser^1
b=D-Ser^1
Boc Boc Boc Boc
H-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OBu^t · 2HCl
(XX)
1equiv HCl
HOBt/DCC
OBu^t Boc Boc Boc Boc
Boc-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Pmp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OBu^t . 3HCl
(XXI)
a=L-Ser^1
b=D-Ser^1
75% TFA
[L-Ser^1, Pmp^9]-β-corticotrophin-(1-24)-tetracosapeptide
[D-Ser^1, Pmp^9]-β-corticotrophin-(1-24)-tetracosapeptide

Scheme III, 3

derivatives (XIX a and b) were purified by crystallization and used for the acylation of the *C*-terminal, partially protected tetradecapeptide ester XX, to give the two tetracosapeptide derivatives XXI a and b. These compounds were purified by countercurrent distribution, and then deprotected with 75% trifluoroacetic acid. Although the risk of formation of alkylated by-products is rather small in the present case (no tryptophan), the use of 75% trifluoroacetic acid was preferred to the higher concentrations which are often used. The resulting trifluoroacetates were finally converted into acetates by a weakly acidic ion exchanger.

Amino acid analyses of acid hydrolysates of the products gave all amino acids in the expected ratios except pentamethylphenylalanine. Either by strong adherence to the matrix of the ion exchange column or by its extremely low solubility in buffer solutions the amino acid is lost during the operation. (The same was found for a sample of the free amino acid.)

III. 3 EXPERIMENTAL SECTION

For details concerning the abbreviations, thin-layer chromatography and the performance of measurements, see the appendices.

Scheme III, 1.

Boc-Pmp-OH (I)

2.90 g (10 mmoles) of H-Pmp-OH.HCl.H_2O were dissolved in 300 ml of a 0.1 N NaOH solution by gentle heating in a water bath under N_2. About 100 ml of dioxan, followed by 25 ml of *t*-butyloxycarbonyl azide[10] (180 mmoles), were added with stirring. With the aid of an autotitrator (Radiometer, Kopenhagen) the pH was kept constant at 11.0 with 1 N NaOH solution. The reaction was carried out in a nitrogen atmosphere to prevent uptake of CO_2. After some 16 hours the slightly turbid solution was extracted twice with ether and acidified with 2 N $KHSO_4$ solution. (The ether extract contained the excess of Boc-N_3 which was recycled.) The product precipitated but dissolved rapidly on adding ether. The water layer was extracted twice with ether, the combined ether layers were washed with water and saturated NaCl solution, dried and evaporated. See table III-1 for the results. (The procedure was worked out first with the D-enantiomer.)

H-Gly-OMe.HCl (II)

This ester was obtained in the normal way with thionyl chloride and methanol[11].
Yield: 99%. M.p.: 177-178°C. TS: Rf = 0.29 (B), = 0.10 (E) (ninh. and R-H).
Analysis:

$C_3H_8NO_2Cl$	Calcd.:	% C 28.70	% H 6.42	% N 11.16	% Cl 28.23
(125.55)	Found :	% C 28.9	% H 6.5	% N 11.5	% Cl 28.6

Table III-1

	Boc-L-Pmp-OH	Boc-D-Pmp-OH
Yield (%)	92.0	91.2
M.p. (^{o}C)	163.0-165.0	163.5-166.0
TS: Rf (B) (R-H)	0.80	0.80
(E) (R-H)	0.80	0.80
$[\alpha]^{21}$ (c = 2.0, MeOH)		
Na 589	-9.6^{o}	+9.8^{o}
Hg 578	-9.9^{o}	+10.4^{o}
546	-11.4^{o}	+11.8^{o}
436	-19.5^{o}	+20.5^{o}
365	-29.9^{o}	+30.9^{o}
$[\alpha]^{21}$ (c = 2.0, HOAc)		
Na 589	-27.0^{o}	+26.8^{o}
Hg 578	-28.2^{o}	+28.3^{o}
546	-32.5^{o}	+32.5^{o}
436	-58.7^{o}	+58.8^{o}
365	-97.9^{o}	+98.1^{o}
Analysis		
$C_{19}H_{29}NO_4$ (335.44)		
% C (68.03)	68.1	67.9
% H (8.71)	8.7	8.8
% N (4.18)	4.1	4.1

Boc-Pmp-Gly-OMe (III)

a) Using triphenyl phosphite according to the method of Mitin[4].

To a solution of 1.883 g (15 mmoles) of II and 1.89 ml (15 mmoles) of *N*-ethylmorpholine in 35 ml of DMF were added 5.032 g (15 mmoles) of I, 1.532 g (22.5 mmoles) of imidazole and 6.981 g (22.5 mmoles) of tri-

phenyl phosphite. The resulting slightly yellow solution was stirred at 40°C for some 18 hours. After cooling, the reaction mixture was poured out into 300 ml of cold water with stirring. After cooling for one hour the precipitate formed was filtered, thoroughly washed with water and diisopropyl ether, and dried. The product was analytically pure. Analytical data are given in table III-2.

b) Mixed anhydride procedure[5].

Boc-Pmp-OH (1.677 g, 5 mmoles) was dissolved in 25 ml of DMF and the solution was cooled at -20°C. An equimolar amount of *N*-ethylmorpholine was added with stirring, followed by 0.67 ml (5 mmoles) of isobutyl chloroformate at -15°C. After 2 minutes a solution of 0.690 g (5.5 mmoles) of II and 0.77 ml (5.5 mmoles) of triethylamine in 10 ml of DMF were added and the reaction mixture was stirred for 10 minutes. (The temperature rose spontaneously to about 5°C.) The solution was concentrated *in vacuo*, and about 130 ml of water were added with stirring. The isolated precipitate appeared to contain some unreacted I. Therefore it was dissolved in ethyl acetate, washed with $NaHCO_3$ solution and water, and dried. After evaporation of the solvent, chromatographically pure III remained.

H-Pmp-Gly-OMe.HCl (IV)

5.370 g (13.2 mmoles) of III were dissolved in 35 ml of a 2 N HCl solution in dry, peroxide-free ethyl acetate with stirring. After 20 minutes the solvent was evaporated. Dry ethyl acetate was added to the residue and the solution was stirred again for half an hour in an open flask. The hydrochloride of the dipeptide ester (IV) crystallized spontaneously. After cooling, the product was filtered: 4.170 g (92.1%) of IV were obtained. M.p.: 139.5-141.0°C. TS: Rf =

Table III-2

	Boc-L-Pmp-Gly-OMe		Boc-D-Pmp-Gly-OMe	
	Mitin's method	mixed anhydride	Mitin's method	mixed anhydride
Yield (%)	93.1	90.0	91.9	90.4
M.p. (^{o}C)	158.5-160.0	159.0-160.0	157.5-159.5	159.0-159.5
TS: Rf (E) (R-H)	0.90	0.90	0.89	0.89
(F) (R-H)	0.76			
$[\alpha]_D^{21}$ (c = 2.0, MeOH)	+4.4^{o}	+4.2^{o}	-4.1^{o}	-4.0^{o}
(c = 2.0, DMF)	-9.7^{o}	-9.6^{o}	+9.3^{o}	+9.5^{o}
Analysis				
$C_{22}H_{34}N_2O_5$ (406.52)				
% C (65.00)	65.0	64.7	64.7	65.3
% H (8.43)	8.4	8.4	8.3	8.5
% N (6.89)	6.8	6.6	6.7	6.7

0.46 (E), = 0.59 (G) (ninh. and R-H). $[\alpha]_D^{21}$ = +129.1° (c = 2.0, MeOH); = +84.4° (c = 2.0, DMF).

Analysis:

$C_{17}H_{27}N_2O_3Cl$	Calcd.:	% C 59.55	% H 7.94	% N 8.17
(342.87)	Found :	% C 59.5	% H 8.2	% N 8.2

Msc-Phe-OH (V)

3.300 g (20 mmoles) of L-phenylalanine were suspended in 25 ml of a mixture of acetonitrile and water (4:1). In the suspension, 5.780 g (20 mmoles) of methylsulphonylethyl *p*-nitrophenyl carbonate (prepared from 2-methylsulphonylethanol and *p*-nitrophenyl chloroformate[2]) were dissolved, and the reaction was started by the addition of 2.8 ml (20 mmoles) of triethylamine. The suspension was stirred overnight and then filtered yielding 210 mg of unchanged phenylalanine (6.4%). The filtrate was concentrated *in vacuo*, diluted with 20 ml of water, acidified to pH 5, and extracted with ether. The aqueous phase was acidified to pH 2, and the resulting biphasic system was heated till a clear solution was obtained. Upon cooling, crystallization set in giving 5.190 g (82.0%) of V. Recrystallization from water gave a product melting at 63°C. Upon drying *in vacuo* over phosphorus pentoxide efflorescence was observed and the melting point rose to 113°C. TS: Rf = 0.37 (E) (R-H). $[\alpha]_D^{22}$ = +6.0° (c = 1.5, HOAc); = -2.6° (c = 1.0, MeOH); = -4.0° (c = 1.0, pyridine).

Analysis

$C_{13}H_{17}NO_6S$	Calcd.:	% C 49.52	% H 5.43	% N 4.44	% S 10.17
(315.34)	Found :	% C 49.5	% H 5.4	% N 4.4	% S 10.2

Msc-Phe-ONp (VI)

630 mg (2 mmoles) of Msc-Phe-OH were dissolved in 5 ml of pure dimethoxyethane, and 306 mg of *p*-nitrophenol were added. The mixture was cooled at 0°C and 413 mg of solid

DCC were carefully added. After 30 minutes the reaction flask was allowed to attain room temperature, left for 1 hour and then put in the refrigerator to complete the crystallization of DCU. The mixture was filtered and the filtrate was evaporated to dryness, giving an oily residue that rapidly crystallized. Recrystallization from methanol gave a product melting at 141-142°C. Yield: 733 mg (84%). TS: Rf = 0.78 (E), = 0.76 (H) (UV and R-H). $[\alpha]_D^{21}$ = -18.7° (c = 1.5, CH_3CN).

Analysis:

$C_{19}H_{20}N_2O_8S$	Calcd.:	% C 52.29	% H 4.62	% N 6.42	% S 7.35
(436.44)	Found :	% C 52.1	% H 4.4	% N 6.6	% S 7.3

Msc-Phe-Arg-OH (VII)

2.530 g (5.8 mmoles) of Msc-Phe-ONp were dissolved in 20 ml of acetonitrile, and 0.912 g (5.22 mmoles, 90% with respect to the activated ester) of arginine, dissolved in 4 ml of water, were added. The mixture was stirred at room temperature, giving a clear solution in about 90 minutes. On chromatography only a trace of arginine was found. The reaction mixture was evaporated, and the residue was diluted with water and filtered to remove unreacted Msc-Phe-ONp. The pH of the water layer was adjusted to 5 using a strongly acidic ion exchanger (H-cycle). After extraction with ether the aqueous phase was concentrated, giving about 40% of pure VII after cooling. A second batch, increasing the yield to 65%, was obtained by heating the evaporated filtrate with ethanol. M.p.: 170-172°C. $[\alpha]_D^{21}$ = -3.3° (c = 1.0, H_2O). TS: Rf = 0.53 (H) (R-H).

Analysis:

$C_{19}H_{27}N_5O_6S.H_2O$	Calcd.:	% C 46.62	% H 6.38
(471.53)	Found :	% C 46.7	% H 6.1
	Calcd.:	% N 14.31	% S 6.55
	Found :	% N 14.4	% S 6.7

The residue from the mother liquor was refluxed with 6 N HCl (20 ml) for 3 hours. After cooling at 0°C, Msc-Phe-OH partially crystallized. It had the same optical rotation as the starting compound. Phenylalanine could not be detected chromatographically in the hydrolysate.

Msc-Phe-Arg-Pmp-Gly-OMe.HCl (VIII)

The best results were obtained with the following procedure: 5.5 mmoles (2.692 g) of the hydrate of VII were dissolved in 25 ml of DMF. The solution was evaporated to dryness and the residue again taken up in 25 ml of DMF. After cooling to -5°C 5.0 mmoles (1.714 g) of HCl.H-Pmp-Gly-OMe in 5 ml of DMF and 11 mmoles (1.486 g) of HOBt, and subsequently at -10°C 5.5 mmoles (1.135 g) of DCC were added. The mixture was stirred for 75 minutes. The temperature was then allowed to rise to room temperature and the mixture was left for another 16 hours. After cooling, the DCU was filtered and the filtrate was dripped out into cold, dry ethyl acetate with stirring. After washing the precipitate with ethyl acetate, about 95% of crude VIII remained. A chromatographically pure product could not be obtained with Sephadex LH-20 and elution with DMF/MeOH (1:1), nor by ion exchange chromatography with Dowex 50X2, nor *via* several crystallizations. By the short-column method of Hunt and Rigby[7], however, the product could be sufficiently purified for further use. TS: Rf = 0.35 (B), = 0.48 (F) (R-H). $[\alpha]_D^{25}$ = -26.1° (c = 0.5, DMF).

H-Phe-Arg-Pmp-Gly-OH (IX)

1.808 g (2.27 mmoles) of the hydrochloride VIII were suspended in 28 ml of pure dioxan and 10 ml of methanol. With vigorous stirring 2.3 ml 4N NaOH (4 equivalents, excess of one equivalent) were added. After 60 seconds the pH was brought to about 2 with HCl, and the reaction mix-

ture was evaporated. The residue was taken up in ethanol and filtered. The pH of the filtrate was brought to 5 with pyridine and the solution was cooled and filtered. Chromatographically homogeneous preparations were obtained when the solid was taken up in acetonitrile-water (1:1) and precipitated with acetonitrile (5-10 times the volume of the solution). Yield: 60-70%. TS: Rf = 0.17 (B), = 0.23 (F), = 0.48 (H) (ninh. and R-H). M.p.: 210-214° (dec.). Elemental analysis, after equilibration with air moisture, indicated that the amount of HCl bound by different preparations varied from 1 to 3 mmoles of HCl per mmole of tetrapeptide.

Scheme III, 2.

Boc-Phe-OH (X)

This compound was obtained by Schnabel's pH Stat method[3]; recrystallization was achieved from diisopropyl ether.

Yield	: 85%	Lit.[3]	91%	[12] 73%
M.p.	: 86-87.5°C		84-86°C	79-80°C
$[\alpha]_D^{21}$	: -4.4°		-4.0°	-0.8°
	(c=1.0,HOAc)		(c=1,HOAc)	(c=4.96,HOAc)
TS	: Rf = 0.07 (A), = 0.70 (B), = 0.29 (G) (R-H)			
Analysis:				
$C_{14}H_{19}NO_4$	Calcd.:	% C 63.38	% H 7.22	% N 5.28
(265.31)	Found :	% C 63.3	% H 7.2	% N 5.3

Boc-Phe-ONSu (XI)

The 1-succinimidyl ester of X was prepared according to Anderson .

Yield	: 79%	Lit.[5]	81%	[13] 88%
M.p.	: 152-153°C		152-153°C	152-153°C
$[\alpha]_D^{21}$	: -21.3°		-19.0°	-20.7°
	(c=2.0,dioxan)		(c=2,dioxan)	(c=2.2,dioxan)

TS : Rf = 0.74 (B), = 0.75 and 0.24 (dec.) (G) (R-H)

Analysis:

$C_{18}H_{22}N_2O_6$	Calcd.:	% C 59.66	% H 6.12	% N 7.73
(362.38)	Found :	% C 59.3	% H 6.3	% N 7.5

H-Arg(NO_2)-OMe.x HCl (XII)

In contradistinction to reports in the literature[14,15] esterification of nitroarginine (obtained by nitration of free arginine with nitric acid and fuming sulphuric acid) with thionyl chloride and methanol did not result in a crystalline compound after working up the evaporated reaction mixture with methanol and ether. The amorphous product appeared to contain about 1.5 equivalents of chlorine which was present as hydrochloric acid. Therefore, the total amount of HCl was neutralized before addition of the activated ester in the next step.

Boc-Phe-Arg(NO_2)-OMe (XIII)

After the usual working up of the reaction mixture (a 10% excess of XII was used) we obtained 76.1% of a chromatographically pure product. M.p. 90-95°C. $[\alpha]_D^{21}$ = -12.7° (c = 1.0, MeOH). TS: Rf = 0.73 (B), = 0.68 (E), = 0.62 (G) (UV and R-H). ε = 14,770 at λ_{max} = 269.0 nm in DMF-0.2 N HCl (1:1, v/v) and ε = 1,020 at λ_{max} = 259.0 nm in TFA. Nitroarginine content: 44% (theoretically 45.6%)[16].

Analysis:

$C_{21}H_{32}N_6O_7$	Calcd.:	% C 52.49	% H 6.71	% N 17.49
(480.52)	Found :	% C 52.4	% H 6.8	% N 17.4

Boc-Phe-Arg(NO_2)-OH (XIV)

Hydrolysis of XIII in dioxan-aqueous NaOH gave the free acid in 90% yield.

M.p. : 114-119°C Lit.[17] 115-120°C

$[\alpha]_D^{21}$: -8.0° (c = 1.0, DMF) -8.5° (c = 1, DMF)
-4.2° (c = 1.0, MeOH)

TS : Rf = 0.57 (B), = 0.24 (E) (UV and R-H)

ε : 15,900 at λ_{max} = 268.5 nm in DMF-0.2 N HCl (1:1, v/v) and

ε : 1,080 at λ_{max} = 259.0 nm in TFA. Nitroarginine content: 46% (theoretically 46.9%)[16].

Analysis:

$C_{20}H_{30}N_6O_7$	Calcd.:	% C 51.49	% H 6.48	% N 18.02
(466.49)	Found :	% C 51.7	% H 6.8	% N 17.7

Boc-Phe-Arg(NO_2)-Pmp-Gly-OMe (XV)

Boc-Phe-Arg(NO_2)-OH (XIV, 3.163 g = 6.78 mmoles) and 0.916 g (6.78 mmoles) of HOBt were dissolved in 25 ml of DMF and the solution was cooled in an ice/salt bath at -13°C. An equimolar amount of DCC (1.401 g) was added with stirring. After half an hour the temperature was brought to 0°C and the reaction mixture was kept at 0°C for 30 minutes. Finally, the mixture was left for one hour at room temperature. A solution of 2.325 g (6.78 mmoles) of IV and 0.855 ml (6.78 mmoles) of *N*-ethylmorpholine in 10 ml of DMF were then added with stirring. After 4 hours a few drops of acetic acid were added, the reaction mixture was cooled and filtered, and the filtrate was poured into 300 ml of water with stirring. After working up, 85% of the crude protected tetrapeptide was obtained. Recrystallization from ethanol gave XV in a yield of 65%. M.p.: 217-217.5°C. $[\alpha]_D^{21}$ = -17.8° (c = 2.0, DMF). TS: Rf = 0.91 (E), = 0.60 (I) (UV and R-H). ε = 16,600 at λ_{max} = 261.5 nm in DMF and ε = 1,400 at λ_{max} = 259.5 nm in TFA. Nitroarginine content: 28% (theoretically 28.4%).

Analysis:

$C_{37}H_{54}N_8O_9.H_2O$	Calcd.:	% C 57.50	% H 7.30	% N 14.50
(772.90)	Found :	% C 57.9	% H 7.4	% N 14.7

Neither the removal of the protective groups one by one by treatment with HCl in ethyl acetate, hydrogenation on palladium/charcoal and saponification, nor the elimination of the protective groups in two steps by hydrogenation in a methanolic solution of hydrochloric acid yielded pure, unprotected IX, because the intermediates resisted crystallization.

Scheme III, 3.

Z-Glu(OBu^t)-His-Phe-Arg-Pmp-Gly-OH (XVI)

In several experiments in which H-Phe-Arg-Trp-Gly-OH. HCl (or its free base) was used as a model compound for the condensation with Z-Glu(OBu^t)-His-N_3, it was found that the presence of one mole of HCl did not influence the yield. The use of half an equimolar excess of the azide increased the yield however, from 50 to 80%.

The condensation finally adopted ran as follows: Z-Glu(OBu^t)-His-N_2H_3*[18] (1.447 g, 2.24 mmoles) was dissolved in 20 ml of DMF. At -15°C 4 equivalents of HCl in EtOAc were added and the hydrazide was converted into the azide by the addition of 0.31 ml (1.2 equiv.) of *t*-butyl nitrite. After 20 minutes at -15°C the added hydrochloric acid was neutralized by the addition of *N*-ethylmorpholine, and the azide solution was mixed with a solution of 942 mg (∿ 1.49 mmoles) of IX in 25 ml of DMF. The mixture was stirred for one day at 0°C and then concentrated to about one sixth of its volume. Dilution with water under stirring gave a gelatinous precipitate which was filtered. For chromatography the conventional systems (B, F, H, J, K, L, M) were unsuitable because of "tailing"; only system N invariably gave well-shaped spots. The product could be purified by intensive washings with water, ethyl acetate and methanol, yielding 70% of XVI.

* Gift from Dr. W. Rittel, Ciba-Geigy A.G., Basle.

M.p. : 210-212°C (dec.)
$[\alpha]_D^{21}$: -9.0° (c = 1.0, 90% HOAc)
TS : Rf = 0.72 (N) (R-H, Pauly and Sakaguchi)

H-Glu(OBu^t)-His-Phe-Arg-Pmp-Gly-OH (XVII)

Hydrogenation of XVI was performed in 90% HOAc with palladium on charcoal as a catalyst. After some 3 hours the catalyst was removed by filtration and the filtrate was concentrated. Methanol was added, and the precipitate was filtered after cooling. The procedure was repeated to complete the deprotection (2 hours). Although a small amount of a by-product of unknown constitution could not be removed, the peptide derivative was used as such in the next stage.

Boc-Ser-Tyr-Ser-Met-Glu(OBu^t)-His-Phe-Arg-Pmp-Gly-OH (XIXa)

300 mg (0.50 mmoles) of XVIIIa*[19] were dissolved in 5 ml of DMF, and the solution was cooled at about -25°C. To the cold solution were subsequently added, with stirring, 0.75 ml 2N HCl in EtOAc, then 0.07 ml of *t*-butyl nitrite (0.60 mmoles), after 15 minutes 0.19 ml of *N*-ethylmorpholine (1.50 mmoles) and finally at -30°C a solution of 338 mg (about 0.3 mmoles) of XVII in 40 ml of DMF. (The pH of the solution of XVII in DMF was brought to about 6 if necessary.) The temperature was allowed to rise to 0°C, and the reaction mixture was stirred for 22 hours. The slightly turbid solution was subsequently filtered and concentrated *in vacuo*. Precipitation of the product with acetonitrile yielded about 95% of the crude decapeptide which was recrystallized from acetonitrile-water (3:2). The mother liquor was treated in the same way. The decapeptide derivative, obtained in 70% overall yield, contained about 5% of the corresponding sulphoxide.

* Gift from Dr. W. Rittel, Ciba-Geigy A.G., Basle.

M.p.: 231-234°C (dec.). $[\alpha]_D^{21}$: +12.8° (c = 0.5, 90% HOAc).
TS : Rf = 0.27 (B), = 0.41 (F), = 0.32 (O) (R-H).

The decapeptide derivative XVb was obtained in the same way using XVIIIb*[20]. After recrystallization the yield was about 60%. It also contained a small amount of the sulphoxide derivative. M.p.: 236-238°C (dec.). TS: Rf = 0.42 (F), = 0.31 (O) (R-H). $[\alpha]_D^{21}$ = -6.4° (c = 0.5, pyridine-water, 1:1).

Boc-Ser-Tyr-Ser-Met-Glu(OBu^t)-His-Phe-Arg-Pmp-Gly-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-OBu^t (XXIa)

Following the procedure of Riniker and Rittel[20] the coupling of XIXa with 1.2 equivalents of XX* was carried out in DMF. Two equivalents of HOBt (instead of HONSu) and 1.5 equivalents of DCC were added, and the reaction was allowed to proceed at 45°C in a N_2 atmosphere. After 18 hours the reaction mixture was cooled and filtered, and the filtrate dripped out into peroxide-free ether with stirring. Without further treatment the crude material (600 mg) was subjected to counter-current distribution in the system: methanol-chloroform-carbon tetrachloride-buffer (7:5:2:4) (buffer composition: 19.25 g of ammonium acetate and 28.5 ml of acetic acid, made up to 1000 ml with water). After 525 transfers (K = 0.09, r_{max} = 42) 237 mg (about 40%) of a chromatographically pure product were obtained. TS (Antec SL 254): Rf = 0.38 (J), = 0.17 (C), = 0.78 (P) (Pauly and R-H).
M.p. : 205°C (dec.)
$[\alpha]_D^{20}$: -37.6° (c = 1.037, MeOH)
UV spectrum: A = 0.67 at λ_{max} = 276.5 nm (concentration: 4.49 mg of XXIa in 5.0 ml 90% HOAc).

* Gift from Dr. W. Rittel, Ciba-Geigy A.G., Basle.

The same procedure was used to obtain the D-Ser^{1} analogue (XXIb). On counter-current distribution with the same system some 60% of pure XXIb was isolated. On thin-layer plates the compound showed the same Rf-values as its L-Ser^{1}-isomer. $[\alpha]_D^{20}$ = -37.4° (c = 0.879, MeOH).
UV spectrum: A = 0.62 at λ_{max} = 276.5 nm (concentration: 4.16 mg of XXIb in 5.0 ml 90% HOAc).

[Pmp^{9}]-β-corticotrophin-(1-24)-tetracosapeptide

The protected tetracosapeptide acetate (XXIa, 170 mg) was dissolved in 17 ml of 75% trifluoroacetic acid (by volume). The slightly turbid solution was kept for 3 hours in an atmosphere of nitrogen in the dark, then cooled and slowly poured into 85 ml of dry peroxide-free ether, which was vigorously stirred. After some 10 minutes the precipitate was filtered by forcing the suspension through a sintered glass funnel by nitrogen pressure. The sticky trifluoroacetate was then dissolved in 0.1 N acetic acid, and the solution was filtered through a column of a weakly basic anion exchanger (Merck II - acetate form). Eluate fractions were pooled until the Folin-Ciocalteux reaction became negative, and the solution was then lyophylized. TA: Rf = 0.52 (P); TC: Rf = 0.47 (P), = 0.40 (Q), = 0.55 (R) (ninh., Pauly and R-H). After equilibration with air moisture the following constants were found: $[\alpha]_D^{20}$ = -83.6° (c = 0.506, 1% HOAc), $[\alpha]_{578}$ = -87.7°, $[\alpha]_{546}$ = -100.0°, $[\alpha]_{436}$ = -175.1° and $[\alpha]_{365}$ = -282.2°.
Amino acid analysis after complete hydrolysis (6 N HCl, 24 hours at 110°C) (theoretical values in parentheses):
Lys 3.76 (4), His 1.06 (1), NH_3 1.40, Arg 2.98 (3), Ser 1.66 (2), Glu 1.02 (1), Pro 3.20 (3), Gly 1.96 (2), Val 3.00 (3, standard), Met 0.98 (1), Tyr 1.90 (2) and Phe 0.93 (1). Peptide content: 75 ± 1% (mean of 3 analyses).
UV spectra: in 0.1 N HCl A = 0.560 at λ_{max} = 276.5 nm (concentration 0.688 mg/ml),

in 0.1 N NaOH A = 0.784 at λ_{max} = 296.0 nm (concentration 0.632 mg/ml).

[D-Ser^1, Pmp^9]-β-corticotrophin-(1-24)-tetracosapeptide

The same procedure was used as in the foregoing preparation. TA: Rf = 0.52 (P); TC: Rf = 0.47 (P), = 0.40 (Q), = 0.55 (R) (ninh. Pauly and R-H).
$[\alpha]_D^{20}$ = -80.7° (c = 0.477, 1% HOAc), $[\alpha]_{578}$ = -84.5°, $[\alpha]_{546}$ = -96.0°, $[\alpha]_{436}$ = -169.0° and $[\alpha]_{365}$ = -271.5°.
Amino acid analysis after complete hydrolysis:
Lys 3.82 (4), His 1.08 (1), NH_3 0.63, Arg 2.74 (3), Ser 1.51 (2), Glu 0.95 (1), Pro 3.12 (3), Gly 1.89 (2), Val 3.00 (3, standard), Met 0.90 (1), Tyr 1.84 (2), Phe 0.97 (1).
UV absorption: in 0.1 N HCl A = 0.533 at λ_{max} = 276.3 nm (concentration 0.662 mg/ml), in 0.1 N NaOH A = 0.770 at λ_{max} = 296.0 nm (concentration 0.634 mg/ml).

REFERENCES

1. Schwyzer, R. and Kappeler, H. (1963) *Helv. Chim. Acta* 46, 1550-1572 and references therein.
2. Tesser, G.I., in preparation.
3. Schnabel, E. (1967) *Justus Liebigs Ann. Chem.* 702, 188-196.
4. Mitin, Y.V. and Glinskaya, O.V. (1969) *Tetrahedron Letters*, 5267-5270.
5. Anderson, G.W., Zimmerman, J.E. and Callahan, F.M. (1967) *J. Amer. Chem. Soc.* 89, 5012-5017.
6. König, W. and Geiger, R. (1971) in Peptides 1969, Proceedings of the 10th European Peptide Symposium (Scoffone, E., ed.) pp. 17-22, North-Holland Publishing Co., Amsterdam; *Chem. Ber.* (1970) 103, 788-798.

7. Hunt, B.J. and Rigby, W. (1967) *Chem. and Ind.* 1868-1869.
8. Anderson, G.W., Zimmerman, J.E. and Callahan, F.M. (1964) *J. Amer. Chem. Soc.* 86, 1839-1842.
9. Honzl, J. and Rudinger, J. (1961) *Coll. Czech. Chem. Comm.* 26, 2333-2344.
10. Carpino, L.A., Giza, C.A. and Carpino, B.A. (1959) *J. Amer. Chem. Soc.* 81, 955-957.
11. Brenner, M. and Huber, W. (1953) *Helv. Chim. Acta* 36, 1109-1115.
12. Anderson, G.W. and McGregor, A.C. (1957) *J. Amer. Chem. Soc.* 79, 6180-6183.
13. Wünsch, E. and Drees, F. (1967) *Chem. Ber.* 100, 816-819.
14. Hofmann, K., Peckham, W.D. and Rheiner, A. (1956) *J. Amer. Chem. Soc.* 78, 238-242.
15. Gibian, H. and Schröder, E. (1961) *Justus Liebigs Ann. Chem.* 642, 145-162.
16. Gamper, J.R. (1962) *Helv. Chim. Acta* 45, 1418-1425.
17. Nicolaides, E., DeWald, H., Westland, R., Lipnik, M. and Posler, J. (1968) *J. Med. Chem.* 11, 74-79.
18. Schwyzer, R. and Kappeler, H. (1961) *Helv. Chim. Acta* 44, 1991-2002.
19. Iselin, B. and Schwyzer, R. (1961) *Helv. Chim. Acta* 44, 169-173.
20. Riniker, B. and Rittel, W. (1970) *Helv. Chim. Acta* 53, 513-519.

CHAPTER IV

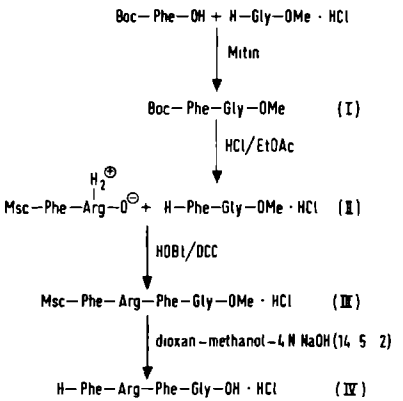
SYNTHESIS OF TWO ACTH-(1-24) ANALOGUES CONTAINING L-PHENYLALANINE IN POSITION 9

IV. 1 INTRODUCTION AND METHOD OF SYNTHESIS

The only difference between the synthesis of the tetracosapeptides indicated in the title and those described in the previous chapter, concerns the preparation of the intermediate consisting of the desired sequence 7-10. This compound has been synthesized before as its acetate by Hofmann *et al.*[1] in their preparation of [Gln^5, Phe^9]-β-corticotrophin-(1-20)-amide in a stepwise procedure in which Arg^8 was induced as ω-nitroarginine.

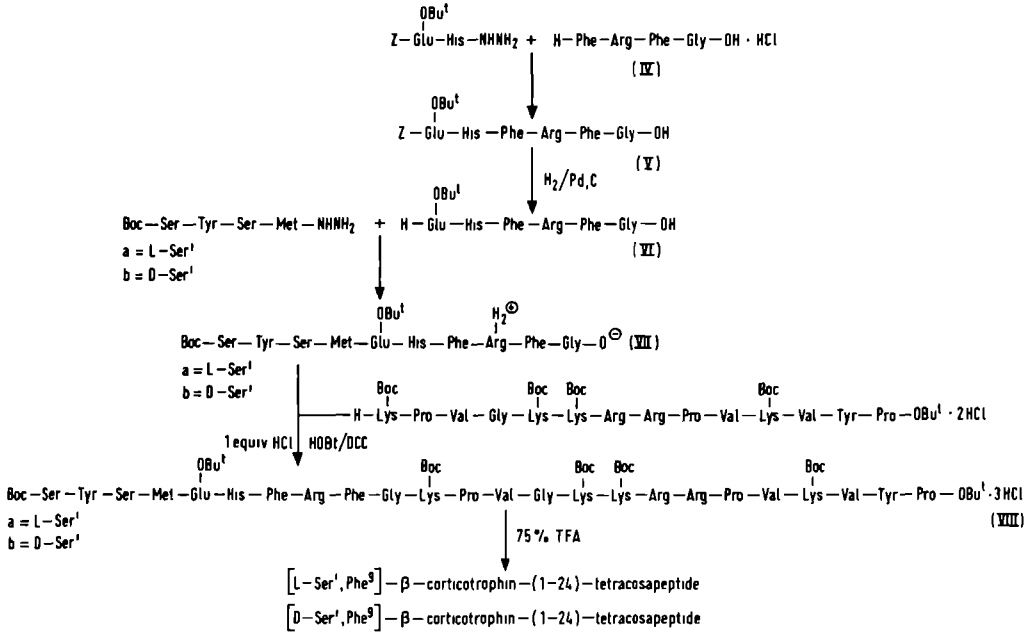
We preferred the fragment condensation technique which has also been used in the synthesis of the pentamethylphenylalanine analogue (Chapter III). The water-soluble product, Msc-Phe-Arg-Phe-Gly-OMe.HCl (III, scheme IV, 1), was deprotected without previous purification. The working up of the reaction mixture was changed in order to obtain only one mole of hydrogen chloride per mole of free tetrapeptide.

Elongation of the tetrapeptide salt (IV) to the protected zwitter-ionic decapeptides (VIIa and b, scheme IV, 2) was carried out in the same way as described in chapter III for the pentamethyl-



Scheme IV, 1

phenylalanine analogues. Both decapeptides were converted into the corresponding protected tetracosapeptide esters (VIIIa, b) with 1-hydroxybenzotriazole and dicyclohexylcarbodiimide as the condensing agent. After counter-current distribution followed by deprotection with trifluoroacetic acid, the resulting trifluoroacetates of the end-products were converted into acetates.


OBu^t
Z–Glu–His–NHNH2 + H–Phe–Arg–Phe–Gly–OH · HCl
(IX)
OBu^t
Z–Glu–His–Phe–Arg–Phe–Gly–OH
(X)
H2/Pd,C
OBu^t
Boc–Ser–Tyr–Ser–Met–NHNH2 + H–Glu–His–Phe–Arg–Phe–Gly–OH
a = L–Ser'
b = D–Ser'
(XI)
OBu^t
H2^⊕
Boc–Ser–Tyr–Ser–Met–Glu–His–Phe–Arg–Phe–Gly–O^⊖ (VII)
a = L–Ser'
b = D–Ser'
Boc
Boc
Boc
Boc
H–Lys–Pro–Val–Gly–Lys–Lys–Arg–Arg–Pro–Val–Lys–Val–Tyr–Pro–OBu^t · 2HCl
1 equiv HCl
HOBt/DCC
OBu^t
Boc
Boc
Boc
Boc–Ser–Tyr–Ser–Met–Glu–His–Phe–Arg–Phe–Gly–Lys–Pro–Val–Gly–Lys–Lys–Arg–Arg–Pro–Val–Lys–Val–Tyr–Pro–OBu^t · 3HCl
a = L–Ser'
b = D–Ser'
(VIII)
75 % TFA
[L–Ser', Phe^9]–β–corticotrophin–(1–24)–tetracosapeptide
[D–Ser', Phe^9]–β–corticotrophin–(1–24)–tetracosapeptide

Scheme IV, 2

IV. 2 EXPERIMENTAL SECTION

For details concerning the abbreviations, thin-layer chromatography and the performance of measurements, see the appendices.

Boc-Phe-Gly-OMe (I)

Boc-Phe-OH and H-Gly-OMe.HCl (see chapter III) were coupled using Mitin's method[2].

Yield : 80.0% Lit.[3] 63-86%

M.p. : 94-95°C 93-95°C

$[\alpha]_D^{25}$: -5.2° (c = 1.0, MeOH) -5.6° (c = 2.3, MeOH)
-10.4° (c = 1.57, DMF) -10.5° (c = 1.3, DMF)

TS : Rf = 0.74 (B), = 0.79 (G) (R-H)

Analysis:

$C_{17}H_{24}N_2O_5$	Calcd.:	% C 60.70	% H 7.19	% N 8.33
(336.39)	Found :	% C 60.6	% H 7.3	% N 8.4

H-Phe-Gly-OMe.HCl (II)

Deprotection was achieved with HCl in dry EtOAc (compare the Pmp-analogue in chapter III).

Yield : 90.5%

M.p. : 137.5-139°C

$[\alpha]_D^{21}$: +35.3° (c = 1.0, MeOH)
+20.9° (c = 1.0, DMF)
+51.7° (c = 1.0, H_2O) Lit.[1] +52.3° (c = 1.8, H_2O)

TS : Rf = 0.39 (B), = 0.60 (G) (ninh. and R-H)

Analysis:

$C_{12}H_{17}N_2O_3Cl$	Calcd.:	% C 52.85	% H 6.28	% N 10.27
(272.73)	Found :	% C 52.9	% H 6.3	% N 10.2

Msc-Phe-Arg-Phe-Gly-OMe.HCl (III)

5.9 mmoles (2.890 g) of Msc-Phe-Arg(H_2^+)-O^-.H_2O (see chapter III) were dissolved in 25 ml of DMF and the solution was

cooled to about -8°C. To this solution were added 5.47 mmoles (1.491 g) of II in 5 ml of DMF and 11.8 mmoles (1.594 g) of HOBt, followed by 5.9 mmoles (1.217 g) of DCC. The mixture was stirred for one hour and the temperature was then allowed to rise to room temperature. After some 18 hours the reaction mixture was cooled and filtered, and the greater part of the filtrate evaporated. Addition of dry ethyl acetate resulted in a gum which could be transformed into a filterable substance by rubbing with ether. 95.0% of III was obtained. The product was deprotected without further purification.

H-Phe-Arg-Phe-Gly-OH.HCl (IV)

1.628 g (2.23 mmoles) of III were dissolved in 28 ml of pure dioxan and 10 ml of methanol. 4 equivalents (excess of one equivalent) NaOH were added as a 4 N solution with stirring. After about one minute the pH was brought to about 7 and the turbid solution was filtered and evaporated. The residue was dissolved in water-acetonitrile (1:1) and HCl was added with stirring until pH 5. To this well-stirred solution, acetonitrile was slowly added (about 5 times the volume of the solution). After one hour the flask was cooled. By filtering and drying 1.10 g of IV were obtained. A chromatographically homogeneous preparation was obtained when the substance was dissolved in a small amount of water-acetonitrile (1:1) and acetonitrile was slowly added to this solution with stirring. Yield: 916 mg of the monohydrate of IV = 70.9%. M.p.: 176-178° (dec. at about 200°C). TS: Rf = 0.25 (F), = 0.55 (N), = 0.40 (P) (ninh. and R-H). $[\alpha]_D^{21}$ = +9.6° (c = 1.08, 90% HOAc), = +5.8° (c = 1.0, H_2O).

Analysis:

$C_{26}H_{36}N_7O_5Cl.H_2O$	Calcd.:	% C 53.83	% H 6.60
(580.09)	Found :	% C 53.6	% H 6.4

Calcd.:	% N 16.90	% Cl 6.12
Found :	% N 16.8	% Cl 6.4

Z-Glu(OBu^t)-His-Phe-Arg-Phe-Gly-OH (V)

The azide coupling leading to V was carried out in exactly the same way as described in chapter III for the Pmp-hexapeptide. Dissolution of the crude product (about 89%) in DMF and precipitation with water (with stirring), followed by cooling, filtering, washings with water and ethyl acetate and drying yielded 883 mg of the dihydrate of V (70.5%). M.p.: 206-207°C. TS: Rf = 0.41 (F), = 0.68 (N), = 0.52 (P) (R-H). $[\alpha]_D^{21}$ = -13.4° (c = 1.0, 90% HOAc) (Perkin-Elmer 241).

Analysis:

$C_{49}H_{63}N_{11}O_{11}\cdot 2H_2O$	Calcd.: % C 57.80	% H 6.63	% N 15.13
(1018.14)	Found : % C 57.8	% H 6.5	% N 15.0

H-Glu(OBu^t)-His-Phe-Arg-Phe-Gly-OH (VI)

Hydrogenation of V was performed in 90% HOAc with palladium on charcoal as a catalyst. After some 5 hours the catalyst was removed by filtration and the filtrate was concentrated. By addition of a small amount of methanol followed by a larger amount of ether, VI was precipitated and isolated by filtration. Dissolution of the substance in a small amount of acetic acid and precipitating it in the same way yielded some 80% of VI. Tlc revealed that a trace of an unknown by-product had remained.

Boc-Ser-Tyr-Ser-Met-Glu(OBu^t)-His-Phe-Arg-Phe-Gly-OH (VIIa)

The same procedure as in the synthesis of the corresponding Pmp-analogue was used. Precipitation of the product was carried out with peroxide-free ethyl acetate. Recrystallization of the crude product (94%) by dissolution in water-acetonitrile (1:1) and precipitation with aceto-

nitrile (5-10 times the volume of the solution) yielded 68% of the decapeptide. M.p.: 205-210°C (dec.). TS: Rf = 0.42 (F), = 0.59 (P) (R-H). $[\alpha]_D^{21}$ = -20.0° (c = 0.41, 90% HOAc) (Perkin-Elmer 241).

The decapeptide derivative VIIb was obtained in the same way. The yield, after recrystallization, was 64%. M.p.: 200-205°C (dec.). TS: Rf = 0.42 (F) (R-H). $[\alpha]_D^{21}$ = -14.6° (c = 0.5, pyridine-water, 1:1) (Perkin-Elmer 241).

Boc-Ser-Tyr-Ser-Met-Glu(OBu^t)-His-Phe-Arg-Phe-Gly-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-OBu^t (VIIIa)

The same procedure as described in chapter III was followed to obtain the protected tetracosapeptide VIIIa. The crude material (400 mg) was subjected to counter-current distribution in the system: methanol-chloroform-carbon tetrachloride-buffer (8:5:2:4) (buffer composition: 19.25 g of ammonium acetate and 28.5 ml of acetic acid, made up to 1000 ml with water). After 260 transfers (K = 0.4, r_{max} = 75) 150 mg (40%) of a chromatographically pure compound were obtained. M.p.: 195-200°C. TS: Rf = 0.22 (B), = 0.42 (C), = 0.44 (F) (R-H, Pauly and Sakaguchi). $[\alpha]_D^{21}$ = -43.8° (c = 0.74, MeOH) (Perkin-Elmer 241). UV spectrum: A = 0.46 at λ_{max} = 277.0 nm (concentration: 2.75 mg of VIIIa in 5.0 ml 90% HOAc).

The D-Ser^1 analogue was obtained in the same way. On counter-current distribution with the same system the product appeared to move much slower than the L-Ser^1 peptide. After 370 transfers a small amount of a by-product was still present; another 300 transfers in a closed circuit gave pure VIIIb (K = 0.22, r_{max} = 22) in about 40% yield. The compound showed the same Rf-values on thin-layer plates as its L-Ser^1 isomer. $[\alpha]_D^{21}$ = -44.0° (c = 1.0,

MeOH) (Perkin-Elmer 241). UV spectrum: A = 0.57 at λ_{max} = 278.0 nm (concentration: 3.50 mg of VIIIb in 5.0 ml 90% HOAc).

[Phe^9]-β-corticotrophin-(1-24)-tetracosapeptide

Deprotection of VIIIa with 75% trifluoroacetic acid and working up with peroxide-free ether yielded the trifluoroacetate of the free tetracosapeptide; conversion into the acetate was performed with a weakly basic anion exchanger (Merck II-acetate form). TC: Rf = 0.46 (M), = 0.53 (P), = 0.62 (R) (ninh., Pauly and R-H).
$[\alpha]_D^{21}$ = -84.6° (c = 0.50, 1% HOAc), $[\alpha]_{578}$ = -88.2°, $[\alpha]_{546}$ = -100.6°, $[\alpha]_{436}$ = -175.0° and $[\alpha]_{365}$ = -282.4° (Perkin-Elmer 241).
Amino acid analysis after complete hydrolysis (6 N HCl, 24 hours at 110°C) (theoretical values in parentheses): Lys 3.72 (4), His 1.06 (1), NH_3 0.94, Arg 3.01 (3), Ser 1.59 (2), Glu 0.98 (1), Pro 3.20 (3), Gly 1.98 (2), Val 3.00 (3, standard), Met 0.97 (1), Tyr 1.89 (2) and Phe 2.03 (2).
Peptide content: 58 ± 1.5% (mean of 3 analyses).
UV spectra: in 0.1 N HCl A = 0.53 at λ_{max} = 274.5 nm (concentration 0.68 mg/ml),
in 0.1 N NaOH A = 0.83 at λ_{max} = 292.3 nm (concentration 0.58 mg/ml).

[D-Ser^1, Phe^9]-β-corticotrophin-(1-24)-tetracosapeptide

The same procedure was used as in the foregoing preparation. TC: Rf = 0.48 (M), = 0.51 (P), = 0.65 (R) (ninh., Pauly and R-H).
$[\alpha]_D^{21}$ = -84.6° (c = 0.50, 1% HOAc), $[\alpha]_{578}$ = -87.8°, $[\alpha]_{546}$ = -100.2°, $[\alpha]_{436}$ = -174.8° and $[\alpha]_{365}$ = -284.0° (Perkin-Elmer 241).

Amino acid analysis after complete hydrolysis:
Lys 3.59 (4), His 0.96 (1), NH_3 0.79, Arg 3.18 (3), Ser 1.65 (2), Glu 1.01 (1), Pro 2.97 (3), Gly 1.96 (2), Val 3.00 (3, standard), Met 1.03 (1), Tyr 1.91 (2), Phe 2.02 (2). Peptide content: 58 $\pm$ 1.5% (mean of 3 analyses).
UV absorption: in 0.1 N HCl A = 0.55 at λ_{max} = 274.4 nm (concentration 0.68 mg/ml),
in 0.1 N NaOH A = 0.89 at λ_{max} = 291.8 nm (concentration 0.58 mg/ml).

REFERENCES

1. Hofmann, K., Andreatta, R., Bohn, H. and Moroder, L. (1970) *J. Med. Chem.* 13, 339-345.
2. Mitin, Y.V. and Glinskaya, O.V. (1969) *Tetrahedron Letters*, 5267-5270.
3. Buis, J.T.W.A.R.M. (1973) Thesis, Nijmegen.

CHAPTER V

SYNTHESIS OF α-MSH ANALOGUES CONTAINING L-PENTAMETHYLPHENYLALANINE OR L-PHENYLALANINE IN POSITION 9

V. 1 INTRODUCTION

Since the amino acid sequence of α-MSH is identical with that of the amino terminal tridecapeptide of ACTH, the protecting groups and condensing agents employed in the synthesis of ACTH-analogues could also be employed in the synthesis of the corresponding MSH-analogues.

A special feature of the composition of α-MSH is, however, the presence of an *N*-acetyl group at the *N*-terminal serine residue. Various methods have been used for its introduction. They include acetylation of the *N*-terminal dipeptide[1], tripeptide[2] or tetrapeptide[3,4] with acetic anhydride[1,3], pentachlorophenyl acetate[2] or *p*-nitrophenyl acetate[4], and subsequent coupling of the acetylated peptide with the remaining *C*-terminal part[2,3] or with suitably chosen fragments[4,5] leading to the desired acetylated tridecapeptide amide. Only Schwyzer has investigated acetylation of the *N*-terminal serine at the tridecapeptide stage[4]. A protected ACTH-(1-10) decapeptide[6] was coupled with the *C*-terminal tripeptide amide in which the lysine side-chain was protected with the acid-

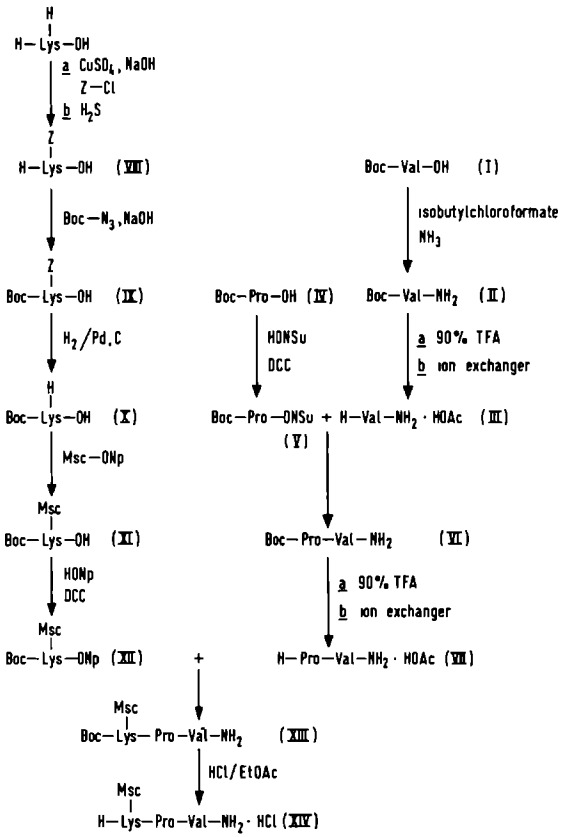
stable phthaloyl group. The acid-labile *t*-butyloxycarbonyl and *t*-butyl ester groups on serine[1] and glutamic acid[5], respectively, were removed and the resulting tridecapeptide derivative was selectively acetylated at the *N*-terminal serine with *p*-nitrophenyl acetate. Treatment of the resulting product with hydrazine acetate gave α-MSH[4].

Since we had the protected *N*-terminal decapeptide at our disposal we followed Schwyzer's strategy but employed the methylsulphonylethyloxycarbonyl group for the protection of the side-chain of lysine in position 11. The group has a very high acid stability and is not attacked by *N*-ethylmorpholine under the conditions needed for acetylation of the *N*-terminal serine residue. Removal of this group was performed in dioxan-methanol-sodium hydroxide solution. Due to irreversible adsorption of a large amount of the product onto the column, purification could not be done by chromatography on carboxymethyl cellulose. This loss of material was also found by Schwyzer[4]. Good results were obtained, however, with Sephadex LH-20 equilibrated with the upper phase of a butanol-acetic acid-water system, using the lower phase for the elution[7].

V. 2 METHOD OF SYNTHESIS

H-Lys(Msc)-Pro-Val-NH_2.HCl (Scheme V, 1)

Boc-Val-NH_2 (II) was prepared from Boc-Val-OH (I) with isobutyl chloroformate and ammonia[4]. After deprotection with 90% trifluoroacetic acid the re-


H
H—Lys—OH
a CuSO4, NaOH
Z—Cl
b H2S
Z
H—Lys—OH (VIII)
Boc—N3, NaOH
Z
Boc—Lys—OH (IX)
H2/Pd.C
H
Boc—Lys—OH (X)
Msc—ONp
Msc
Boc—Lys—OH (XI)
HONp
DCC
Msc
Boc—Lys—ONp (XII)
Boc—Val—OH (I)
isobutylchloroformate
NH3
Boc—Pro—OH (IV)
Boc—Val—NH2 (II)
HONSu
DCC
a 90% TFA
b ion exchanger
Boc—Pro—ONSu + H—Val—NH2 · HOAc (III)
(V)
Boc—Pro—Val—NH2 (VI)
a 90% TFA
b ion exchanger
H—Pro—Val—NH2 · HOAc (VII)
+
Msc
Boc—Lys—Pro—Val—NH2 (XIII)
HCl/EtOAc
Msc
H—Lys—Pro—Val—NH2 · HCl (XIV)

Scheme V, 1

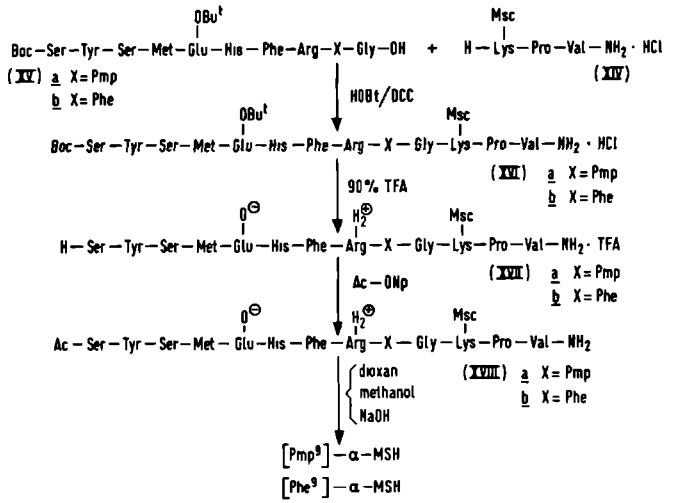
sulting trifluoroacetate was converted into the acetate with a weak basic anion exchanger. Since it is known[4] that coupling of the product III with *t*-butyloxycarbonyl-L-proline (IV) *via* the mixed anhydride procedure is only possible with low yield, the coupling was effected with the 1-succinimidyl ester of IV. Deprotection of the dipeptide derivative VI with 90% trifluoroacetic acid, followed by conversion of the trifluoroacetate into the acetate, yielded chromatographically pure prolyl-valine amide acetate (VII).

The introduction of the *t*-butyloxycarbonyl group into ε-benzyloxycarbonyl-L-lysine (VIII), synthesized by benzyloxycarbonylation of the copper complex of lysine, was carried out according to the method of Schnabel[8]. After hydrogenation with palladium on charcoal as a catalyst, the methylsulphonylethyloxycarbonyl group was introduced in the ε-amino group *via* its *p*-nitrophenyl ester[9], and the protected lysine derivative XI was converted into an activated ester (XII) in the conventional way.

Coupling of XII with VII gave the water-soluble protected tripeptide XIII in fairly good yield. To eliminate the *t*-butyloxycarbonyl group it was treated with hydrogen chloride in ethyl acetate resulting in the desired product XIV as a hydrochloric acid salt.

[*Pmp*^{9}]- *and* [*Phe*^{9}]-α-*MSH* (Scheme V, 2)

The synthesis of the decapeptide XVa and b has



Scheme V, 2

been described in chapters III and IV, respectively.

They were coupled with XIV with the aid of 1-hydroxybenzotriazole and dicyclohexylcarbodiimide[10] in dimethylformamide as the solvent under nitrogen. A chromatographically homogeneous product could not be obtained with Sephadex LH-20 (eluent dimethylformamide-methanol 1:1) nor with Kieselgel 60 (chloroform-methanol 1:1 as eluent). However, after cleavage of the acid-labile protecting groups from XVI (a and b) with 90% trifluoroacetic acid the resulting products XVIIa and b could well be purified

by precipitation from a dimethylformamide solution with ethyl acetate. The acetylation was conducted with two equivalents of *p*-nitrophenyl acetate. XVIIIa and b could then be precipitated with peroxide-free ether. They were converted into the crude α-MSH analogues by treatment with a mixture containing dioxan, methanol and sodium hydroxide solution (3:1:1). Pure [Pmp^{9}]- and [Phe^{9}]-α-MSH were obtained by chromatography on Sephadex LH-20 equilibrated with the upper phase of the system 1-butanol-acetic acid-water (2:1:10), using the lower phase for elution.

V. 3 EXPERIMENTAL SECTION

For details concerning abbreviations, thin-layer chromatography and performance of measurements, see appendices.

Scheme V, 1.

Boc-Val-OH (I)

This compound was prepared according to Schnabel's method[8] and was obtained in about 90% yield.

M.p. :	72.5-74.5°C	Lit.[8] 72-73°C	[11]77-79°C	[12]80°C
$[\alpha]_D^{21}$:	-6.7° (c = 1.3, HOAc)	+6.0° (578 nm, c = 1, HOAc)	-5.8° (c = 1.2, HOAc)	-6.9° (c = 2, HOAc)

TS : Rf = 0.36 (G), = 0.43 (S) (R-H)

Boc-Val-NH_2 (II)

The same procedure as described by Schwyzer[4] was used for this preparation.

Yield: 78% Lit.[4] 68%

M.p. : 155.5-157°C 156-157°C

$[\alpha]_D^{21}$: -0.5° (c = 1.5, MeOH)
-13.4° (c = 1.5, HOAc)

TS : Rf = 0.67 (G), = 0.63 (S) (R-H)

H-Val-NH_2.CH_3COOH (III)

Treatment of II with 90% TFA at room temperature for 30 minutes followed by evaporation of the solvent yielded a yellow oil which crystallized on trituration with ethyl acetate; yield 87%. The white substance had m.p. 123-124°C (Lit.[4]: 91.6% H-Val-NH_2.TFA with m.p. 124-125°C). TS: Rf = 0.10 (E) (R-H). Dissolution in 0.1 N acetic acid followed by filtration of the solution through a column of a weak anion exchanger (Merck II, acetate form) and evaporation

of the filtrate yielded III with m.p. 95-100°C, $[\alpha]_D^{21}$ = +36.6° (c = 1.0, HOAc).

Boc-Pro-OH (IV)

Schnabel's pH Stat method[8] was used to prepare IV.

Yield:	94% Lit.[8]	96%	11 55%	12 -
M.p. :	135-136°C	136-137°	136-137°	133-134°
$[\alpha]_D^{21}$:	-62.0° (c = 1.05, HOAc)	-68.5° (578 nm, c = 1, HOAc)	-60.2° (c = 2, HOAc)	-60.8° (c = 2.0, HOAc)

TS : Rf = 0.67 (E), = 0.32 (G) (R-H)

Boc-Pro-ONSu (V)

3.01 g of IV were dissolved in 30 ml of pure dimethoxyethane. 1.61 g of *N*-hydroxysuccinimide were added with stirring and then 3.18 g of DCC (10% excess) at 0°. After some 15 hours the solution was filtered and the evaporated filtrate was crystallized from isopropyl alcohol.

Yield:	86% Lit.[13]	74%	14 87%	
M.p. :	133-134°C	135-136°	134-136°	
$[\alpha]_D^{21}$:	-55.0° (c = 1.0, dioxan)	-55.3° (c = 2, dioxan)	-55° (c = 1, dioxan)	

TS : Rf = 0.65 and 0.14 (dec.) (A) (R-H)
= 0.76 and 0.35 (dec.) (E) (R-H)

Boc-Pro-Val-NH_2 (VI)

Boc-Pro-ONSu (10 mmoles) and H-Val-NH_2-HOAc (10.5 mmoles) were dissolved in 30 ml of DMF. One equivalent of *N*-ethylmorpholine was added and the solution was left overnight at room temperature. After evaporation of the solvent the residue was dissolved in ethyl acetate and extracted with water, 5% $KHSO_4$ solution, water, 5% $NaHCO_3$ solution and again with water. The organic layer was dried with Na_2SO_4, filtered and evaporated. The residue was crystallized from ether.

Yield: 81% Lit.[4] 44.1%
M.p. : 84-86°C 84°
$[\alpha]_D^{21}$: -14.8° (c = 0.5, DMF) -
TS : Rf = 0.25 (A), = 0.73 (E) (R-H)

H-Pro-Val-NH_2.CH_3COOH (VII)

2.24 g of VI (7.2 mmoles) were dissolved in 90% TFA. After 20 minutes at room temperature, the solution was evaporated and the resulting oil crystallized from ethyl acetate/ether. Yield: 89% of the chromatographically pure trifluoroacetate of H-Pro-Val-NH_2. M.p. 167-168°C (Lit.[4]: 163-165°C).

The trifluoroacetate was converted into the acetate as described for the preparation of II. After evaporation of the solvent the residue was crystallized from ether; the product VII was obtained in a quantitative yield.

M.p. : 126-127°C Lit.[4] 137-138°C [15]138-140°C
$[\alpha]_D^{21}$: -62.4° (c = 0.55, dioxan) - -54.6° (c =
-48.8° (c = 1.0, HOAc) 1.02, dioxan)
TS : Rf = 0.15 (B), = 0.12 (C) (ninh. and R-H)

H-Lys(Z)-OH (VIII)

N^{ε}-carbobenzoxylation of lysine was performed *via* the copper complex of the amino acid as described by Tesser[16]. The blue complex of the product was obtained in 90% yield. The removal of copper with hydrogen sulphide was nearly quantitative and gave the pure product VIII.

M.p. : 258-261°C Lit.[17] 278-280°C [18]*ca.* 235°C
$[\alpha]_D^{21}$: +14.8° (c = 1.65, +17.3° (c = 2, +14.0° (c = 2.86,
2N HCl) 2N HCl) dil. HCl)
TS : Rf = 0.37 (B), = 0.86 (L) (ninh. and R-H)

Boc-Lys(Z)-OH (IX)

The introduction of the Boc-group in VIII was achieved using the pH Stat method[8]. After working up as usual, the

product was obtained as a nearly colourless oil in 90% yield. TS: Rf = 0.38 (G) (R-H). $[\alpha]_D^{21}$ = -11.6° (c = 3.5, DMF). Lit.[19]: $[\alpha]_D^{24}$ = -11.5° (c = 5, DMF).

Boc-Lys-OH (X)

IX was hydrogenated in 90% methanol with Pd/C as the catalyst. After filtration of the catalyst and evaporation of the filtrate, the residue could be crystallized from ethanol.

Yield:	85%	Lit.[4] 86%	[20]
M.p. :	200-201°C	204-205°C	201.5-203°C
$[\alpha]_D^{21}$:	-10.1°	-	-10.7°
	(c = 1.0, HOAc)		(c = 0.85, HOAc)

TS : Rf = 0.26 (B), = 0.79 (L) (ninh. and R-H)

Boc-Lys(Msc)-OH (XI)

To a suspension of 10 mmoles (2.46 g) of X in 15 ml of acetonitrile-water (4:1), 10 mmoles of Msc-ONp[9] (2.89 g) and 10 mmoles (1.4 ml) of triethylamine were added with stirring. After 5 minutes another mmole of Msc-ONp and of Et_3N were added to the now clear solution. The solution was left for half an hour and then evaporated. Water was added to the thick oil. With a few drops of a 2 N $KHSO_4$ solution the pH was adjusted to 5 and the water layer was extracted three times with ether. On acidification of the colourless water layer to pH 3 with 2N $KHSO_4$ solution an oil separated. This was extracted with ethyl acetate, the organic layer dried over Na_2SO_4, filtered and evaporated leaving a thick, colourless oil which started to crystallize spontaneously after a few days. Addition of ethyl acetate/petroleum ether completed the crystallization and gave 92% of a white product melting at 84.5-86°C. TS: Rf = 0.52 (B), = 0.72 (C), = 0.40 (E), = 0.87 (L) (R-H). $[\alpha]_D^{21}$ = +3.6° (c = 2.0, EtOAc).

Analysis:

$C_{15}H_{28}N_2O_8S$	Calcd.:	% C	45.44	% H	7.12	% N	7.07	% S	8.09
(396.46)	Found :	% C	45.2	% H	7.3	% N	7.0	% S	8.2

Boc-Lys(Msc)-ONp (XII)

1.59 g of Boc-Lys(Msc)-OH (XI) (4 mmoles) and 668 mg (10% excess) of HONp were dissolved in 20 ml of dry ethyl acetate. At 0°C 904 mg of DCC (10% excess) were added and the mixture was stirred at 0°C for half an hour and then at room temperature for some 15 hours. After filtration of the DCU, the filtrate was evaporated and the resulting oil crystallized from ether. Recrystallization from isopropyl alcohol gave 87% of white XII. M.p.: 104.5-106°C. TS: Rf = 0.66 (B), = 0.93 (C), = 0.71 (G) (R-H). $[\alpha]_D^{21}$ = -20.9° (c = 1.0, EtOAc).

Analysis:

$C_{21}H_{31}N_3O_{10}S$	Calcd.:	% C	48.73	% H	6.04	% N	8.12	% S	6.20
(517.55)	Found :	% C	49.1	% H	6.1	% N	8.1	% S	6.2

Boc-Lys(Msc)-Pro-Val-NH$_2$ (XIII)

To a solution of 2.47 mmoles (678 mg) of VII in DMF, 2.25 mmoles (1.15 g) of XII were added with stirring. The yellow-coloured solution was supplied with 220 µl of Et_3N which intensified the colour. After standing overnight, tlc revealed that all Boc-Lys(Msc)-ONp had been converted. Evaporation of the solvent left a yellow oil which solidified on addition of dry ether. The product was filtered, washed with ether and dissolved in ethyl acetate. The solution was extracted with $KHSO_4$ solution and a saturated NaCl solution, then dried on Na_2SO_4, filtered and evaporated. Rubbing of the amorphous residue with ether gave a water-soluble white, but still amorphous product in 88% yield, melting at about 76°C. TS: Rf = 0.42 (B), = 0.80 (C), = 0.61 (E) (R-H). $[\alpha]_D^{21}$ = -57.4° (c = 1.0, MeOH).

Analysis:

$C_{25}H_{45}N_5O_9S.H_2O$	Calcd.:	% C 49.98	% H 7.88
(609.74)	Found :	% C 49.7	% H 7.8
	Calcd.:	% N 11.66	% S 5.33
	Found :	% N 11.2	% S 5.3

H-Lys(Msc)-Pro-Val-NH_2.HCl (XIV)

Removal of the α-amino protecting group from XIII was carried out with HCl in dry ethyl acetate. After one hour the precipitate was filtered and washed with ethyl acetate. A solution in water attained pH 3.5-4 indicating that more than 1 equivalent of HCl was present. Therefore, the product was recrystallized from propanol-2. After filtration and washing with cold propanol-2 and ether a 77% yield of XIV was obtained with m.p. 73°C. TS: Rf = 0.10 (C), = 0.20 (F), = 0.29 (T) (ninh. and R-H). $[\alpha]_D^{21}$ = -36.8° (c = 1.17, MeOH).

Analysis:

$C_{20}H_{38}N_5O_7SCl.\frac{1}{2}H_2O$	Calcd.:	% C 44.72	% H 7.32	% N 13.04
(537.08)	Found :	% C 44.9	% H 7.5	% N 12.5
	Calcd.:	% S 5.97	% Cl 6.60	
	Found :	% S 6.1	% Cl 6.5	

Scheme V, 2.

Boc-Ser-Tyr-Ser-Met-Glu(OBu^t)-His-Phe-Arg-X-Gly-Lys(Msc)-Pro-Val-NH_2 (XVI)

a) X = Pmp

To a suspension of 80 mg of XVa (about 0.054 mmoles) in 1 ml of pure pyridine were added 0.11 mmoles of XIV (59.1 mg) and 0.11 mmoles of HOBt (14.9 mg) followed by 1.5 equivalents of DCC (0.081 mmoles = 16.7 mg). The reaction mixture was stirred under nitrogen at 45°C during 19 hours. It was then cooled for some hours to complete the precipitation of DCU which was removed by centrifu-

gation. The desired tridecapeptide was precipitated from the supernatant by addition of peroxide-free ether. It was washed with ether, then water and again with ether. For further purification, the dry peptide was dissolved in DMF, the same amount of MeOH was added to the solution and the peptide was again precipitated with peroxide-free ether. After centrifugation the pellet was washed once with ether. Yield: 69 mg (64%). TS: Rf = 0.35 (F), = 0.64 (P). TC: Rf = 0.90 (M) (R-H). Tlc revealed that a trace of the corresponding sulphoxide was present in the product.

b) X = Phe

0.06 mmoles (85 mg) of XVb, 0.12 mmoles (63.4 mg) of XIV, 0.12 mmoles (16.2 mg) of HOBt, 0.09 mmoles (18.6 mg) of DCC and 1 ml of pure pyridine were stirred under nitrogen at 45°C for about 17 hours. The reaction mixture was worked up as described in the previous preparation, giving 96 mg of crude XVIb. By sequentially washing with water, methanol and ether it was partially purified. The last traces of the tripeptide derivative (XIV) and the sulphoxide of XVIb could not be completely removed, however, neither by chromatography on Sephadex LH-20 nor on Kieselgel 60. Yield: 74%. TS: Rf = 0.39 (F), = 0.66 (P). TC: Rf = 0.82 (F), = 0.85 (P) (R-H).

H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-X-Gly-Lys(Msc)-Pro-Val-NH_2.TFA (XVII)

a) X = Pmp

60 mg of XVa were dissolved in 1 ml of 90% TFA under nitrogen. After 15 minutes peroxide-free ether was added (10 ml). The precipitate formed was isolated by centrifugation and washed with peroxide-free ether and peroxide-free ethyl acetate. It was then dissolved in DMF and

precipitated again with peroxide-free ethyl acetate (about twice the volume of DMF). After centrifugation and several washings with peroxide-free ethyl acetate the product was dried: 54 mg of XVIIa (94%) were obtained. TS: Rf = 0.07 (F), = 0.41 (M). TC: Rf = 0.76 (M) (ninh. and R-H).

b) X = Phe

83 mg of XVIb were deprotected as described above. Before the addition of ether a small amount of DCU was removed by centrifugation. The isolation and purification of XVIIb were carried out in the same way as described for XVIIa. Yield: 60 mg (75%). TS: Rf = 0.06 (F), = 0.44 (M). TC: Rf = 0.70 (M) (ninh. and R-H).

Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-X-Gly-Lys(Msc)-Pro-Val-NH_2 (XVIII)

a) X = Pmp

Acetylation of the *N*-terminal serine was carried out with *p*-nitrophenyl acetate according to the method of Schwyzer *et al.*[4]. To that end 48 mg of XVIIa were dissolved in 0.5 ml of pyridine and 1.0 ml of DMF. One equivalent of *N*-ethylmorpholine (3.2 µl) was added followed by 2 equivalents of Ac-ONp (9.1 mg). After 18 h at room temperature the product was precipitated by addition of peroxide-free ether, isolated by centrifugation and washed with peroxide-free ether and ethyl acetate until the supernatant was completely free of *p*-nitrophenol. Yield: 46 mg (99%). TS: Rf = 0.14 (F), = 0.50 (M) (R-H).

b) X = Phe

59 mg (0.032 mmoles) of XVIIb were dissolved in 1 ml of pyridine and 0.2 ml of DMF. One equivalent (0.004 ml) of *N*-ethylmorpholine was added followed by 0.062 mmoles

(11.2 mg) of Ac-ONp. The product was isolated as described for XVIIIa and further purified by dissolution in DMF and precipitation with peroxide-free ethyl acetate and ether. 51 mg of XVIIIb (91%) were isolated. TS: Rf = 0.26 (F), = 0.50 (M) (R-H).

[Pmp^9]-α-MSH

45 mg (0.024 mmoles) of XVIIIa were dissolved in a mixture of 0.7 ml of dioxan, 0.25 ml of methanol and 0.25 ml of water. 4 equivalents of NaOH (24 µl of a 4N solution) were added, an excess of one equivalent. The solution became immediately yellow-coloured. After 2 minutes 2N HCl was added until pH 5 and the reaction mixture was then evaporated. The residue was dissolved in some ml of the lower phase of the system 1-butanol-acetic acid-water (2:1:10) and the solution was evaporated again. After dissolution in as little of the lower phase as possible, the solution was chromatographed on a column (39 x 2.0 cm) of 31 g of Sephadex LH-20 which was equilibrated with the upper phase of the described solvent system before use. Elution with the lower phase gave 15.2 mg of pure [Pmp^9]-α-MSH and 15.2 mg of a not quite homogeneous sample containing the product and the corresponding sulphoxide (about 20%). Physical constants of the pure compound were: TS: Rf = 0.09 (F), = 0.40 (M) (Pauly, ninh. and R-H). TC: Rf = 0.77 (M) (ninh. and R-H). $[\alpha]_D^{21}$ = -49.7° (c = 1.0, 10% HOAc), $[\alpha]_{578}$ = -52.1°, $[\alpha]_{546}$ = 59.2°, $[\alpha]_{436}$ = -103.8° and $[\alpha]_{365}$ = -169.5°. Amino acid analysis after complete hydrolysis (6N HCl, 24 hours at 110°C) (theoretical values in parentheses): Lys 1.05 (1), His 0.98 (1), NH_3 1.92 (1), Arg 0.95 (1), Ser 1.77 (2), Glu 1.05 (1), Pro 1.01 (1), Gly 1.05 (1), Val 1.00 (1, standard), Met 1.14 (1), Tyr 0.93 (1) and Phe 1.05 (1). Pmp was not detectable (see chapter III). Peptide content: 73 ± 3% (mean of 3 analyses).

[Phe^9]-α-MSH

1) 37 mg (0.021 mmoles) of XVIIIb were suspended in 0.7 ml of dioxan and 0.25 ml of methanol. 4 equivalents (21 µl) of 4N NaOH were added giving instantaneously a yellow colour caused by traces of *p*-nitrophenol or *p*-nitrophenyl acetate. For complete dissolution 0.4 ml of water were then added to the reaction mixture. After 2 minutes 0.2 ml of Dowex 50X2 (about 100% excess) were added. The yellow colour disappeared rapidly. After centrifugation the ion exchanger was washed with water and diluted ammonia (the supernatant of the latter washing was yellow). The combined fractions were evaporated, the residue dissolved in 0.01 M NH_4OAc (pH 6.5) and the solution subjected to chromatography on Carboxymethyl cellulose (Serva Feinbiochemica) (Column: 20.5 x 1.8 cm). Elution with about 550 ml of 0.01 M NH_4OAc (pH 6.5) followed by 150 ml of 0.025 M NH_4OAc (pH 6.5) resulted in a considerable loss (about 50%) of material. The purity of the tridecapeptide was hardly improved. Washing of the contents of the column with 0.6 M NH_4OAc resulted in a few mg of impure [Phe^9]-α-MSH.

2) 42 mg (0.024 mmoles) of XVIIIb were deprotected as described above. After isolation the crude compound was dissolved in the lower phase of the system 1-butanol-acetic acid-water (2:1:10), the solvents were evaporated and the residue taken up in as little of the lower phase as possible. The solution was brought on a column of Sephadex LH-20 (32 x 1.8 cm) which was equilibrated with the upper phase of the solvent system. Elution with the lower phase resulted in 7.5 mg of pure [Phe^9]-α-MSH and 14.9 mg of a chromatographically not quite homogeneous product (containing all of the sulphoxide derivative). TS: Rf = 0.39 (M) (Pauly, ninh. and R-H). TC: Rf = 0.76 (M) (ninh. and R-H).
$[\alpha]_D^{21} = -55.2^o$ (c = 0.50, 10% HOAc), $[\alpha]_{578} = -57.6^o$,

$[\alpha]_{546} = -66.0^{o}$, $[\alpha]_{436} = -114.6^{o}$, $[\alpha]_{365} = -187.0^{o}$. Amino acid analysis after complete hydrolysis (6N HCl, 24 hours at 110°C) (theoretical values in parentheses) Lys 0.94 (1), His 0.98 (1), NH_3 1.28 (1), Arg 1.06 (1), Ser 1.67 (2), Glu 0.95 (1), Pro 0.95 (1), Gly 1.08 (1), Val 1.00 (1, standard), Met 1.05 (1), Tyr 1.08 (1) and Phe 2.17 (2). Peptide content: 70 ± 2% (mean of 3 analyses).

REFERENCES

1. Hofmann, K., Thompson, T.A., Yajima, H., Schwartz, E.T. and Inouye, H. (1960) *J. Amer. Chem. Soc.* 82, 3715-3721.
2. Nishimura, O. and Fujino, M. (1972) *J. of the Takeda Research Lab.* 31, 293-301.
3. Yajima, H., Kawasaki, K., Okada, Y., Minami, H., Kubo, K. and Yamashita, I. (1968) *Chem. Pharm. Bull.* 16, 919-928.
4. Schwyzer, R., Costopanagiotis, A. and Sieber, P. (1963) *Helv. Chim. Acta* 46, 870-889.
5. Hofmann, K., Yajima, H. and Schwartz, E.T. (1960) *J. Amer. Chem. Soc.* 82, 3732-3737.
6. Schwyzer, R. and Kappeler, H. (1961) *Helv. Chim. Acta* 44, 1991-2002.
7. Geiger, R., König, W. and Langner, D. (1973) Ger. Offen. 2,212,695 (Cl. C 07c, A 61 k).
8. Schnabel, E. (1967) *Justus Liebigs Ann. Chem.* 702, 188-196.
9. Tesser, G.I. in preparation.
10. König, W. and Geiger, R. (1971) in Peptides 1969, Proceedings of the 10th European Peptide Symposium (Scoffone, E., ed.) pp. 17-22, North-Holland Publishing Co., Amsterdam; *Chem. Ber.* (1970) 103, 788-798.

11. Anderson, G.W. and McGregor, A.C. (1957) *J. Amer. Chem. Soc.* 79, 6180-6183.
12. Bayer, E., Jung, G. and Hagenmaier, H. (1968) *Tetrahedron* 24, 4853-4860.
13. Anderson, G.W., Zimmerman, J.E. and Callahan, F.M. (1964) *J. Amer. Chem. Soc.* 86, 1839-1842.
14. Beyerman, H.C., Boers-Boonekamp, C.A.M. and Maassen van den Brink-Zimmermannová, H. (1968) *Rec. Trav. Chim.* 87, 257-273.
15. Hofmann, K., Yajima, H., Liu, T., Yanaihara, N., Yanaihara, C. and Humes, J.L. (1962) *J. Amer. Chem. Soc.* 84, 4481-4486.
16. Tesser, G.I. (1961) Thesis, Groningen.
17. Ledger, R. and Stewart, F.H.C. (1965) *Aust. J. Chem.* 18, 933-935.
18. Costopanagiotis, A.A., Handford, B.O. and Weinstein, B. (1968) *J. Org. Chem.* 33, 1261-1264.
19. Tesser, G.I. and Nivard, R.J.F. (1964) *Rec. Trav. Chim.* 83, 53-66.
20. Fletcher, G.A. and Jones, G.T. unpublished results.

CHAPTER VI

ELECTRON DONOR PROPERTIES OF TRYPTOPHAN PEPTIDES AND CORRESPONDING PENTAMETHYLPHENYLALANINE AND PHENYLALANINE PEPTIDES

VI. 1 GENERAL INTRODUCTION

According to Foster[1] the term "complex" in organic chemistry implies substances which are formed by interaction of two or more component molecules (and/or ions), which may have an independent crystal structure, and which will dissociate into their components, at least partially, in the vapour phase and on dissolution. The complexes discussed in this chapter result from the weak interaction between electron donors and electron acceptors (charge-transfer or electron donor-acceptor complexes).

When two widely separated molecules are brought together, the van der Waals energy (W_o) is released until an equilibrium distance has been reached. When the components are a donor (D) and an acceptor (A) molecule the equilibrium distance will be shorter as a consequence of intermolecular resonance. Dependent on the nature of the components the distance is between 3.2 and 3.5 Å. The resulting complex is mainly described by a "no bond" wave function ψ_{D---A}. However, when it absorbs the energy $h\nu$, an excited state is reached, described mainly

by a "dative" wave function $\psi_{D^+---A^-}$ (fig. VI, 1). The energy necessary for this transition is usually smaller than that for promoting an electron in one of the components D and A; the transition gives rise to a characteristic charge-transfer band at the long wavelength side of the spectrum[2,3].

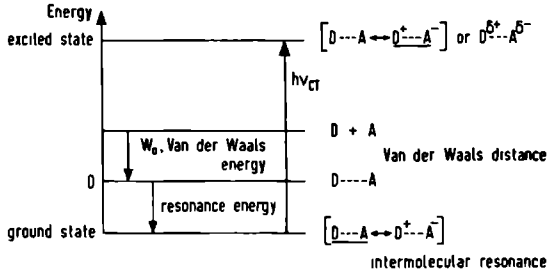


Fig. VI, 1

A number of properties of CT (charge-transfer) complexes can be inferred from the UV-visible absorption characteristics. In our case we are interested in the association constant K of the equilibrium $D + A \underset{}{\overset{K}{\rightleftharpoons}} D^{\delta+}---A^{\delta-}$ and the apparent extinction coefficient ε_{da} of the CT absorption. According to Foster *et al.*[4], K and ε_{da} for a 1:1 complex can be determined with the formula

$$\frac{A_{da}}{C_d \cdot C_a} = -K \cdot \frac{A_{da}}{C_d} + K \cdot \varepsilon_{da}$$

which is valid when $C_a >> C_d$. A_{da} is the observed CT absorption, corrected for residual absorption of donor and acceptor, and C_d and C_a are initial con-

centrations of donor and acceptor, respectively, corrected for volume expansion.

When $\frac{A_{da}}{C_d \cdot C_a}$ is plotted against $\frac{A_{da}}{C_d}$ a linear relationship is to be expected with K as the gradient and $K.\varepsilon_{da}$ as the intercept. In all measurements values for $\frac{A_{da}}{C_d \cdot C_a}$ and $\frac{A_{da}}{C_d}$ at five or six wavelengths in the CT area were subjected to least squares analysis, without resource to graphical evaluation, in order to evaluate K and $K.\varepsilon_{da}$. Extinction coefficients (ε^*) were calculated from $\frac{K.\varepsilon_{da}}{\overline{K}}$ ($\overline{K}$ is the average of K-values found at the various wavelengths). A procedure for the evaluation of ε^*_{max} (to be determined from $\frac{(K.\varepsilon_{da})_{\lambda_{max}}}{\overline{K}}$) can be deduced from the following argument:

It may be presupposed that the position of the maximum in the measured spectra (after correction for residual donor and acceptor absorption) agrees with λ_{max}. The value for $K.\varepsilon$ at λ_{max} can then be determined by reading the absorption (from spectra obtained at the highest concentrations) at one of the measuring-points near λ_{max} (= A_m) and at λ_{max} (= A_{max}). Then: $(K.\varepsilon)_{\lambda_{max}} = \frac{A_{max}}{A_m} . (K\varepsilon)_{\lambda_m}$.

From this ε^*_{max} can be calculated with:

$\varepsilon^*_{max} = \frac{(K.\varepsilon)_{\lambda_{max}}}{\overline{K}}$ (the error in ε^*_{max} depends on the error in $\overline{K}$). $\overline{K}$ was also used to determine the maximum saturation (S_{max}) attained, *i.e.* the fraction of complexed donor at the highest acceptor concentration employed:

$$S_{max} = \frac{C_{da}^{max}}{C_d} = \frac{\overline{K}.C_a^{max}}{1 + \overline{K}.C_a^{max}} \quad (\text{for } C_a^{max} >> C_d)$$

VI. 2 THE ELECTRON ACCEPTOR

In investigations into a possible relationship between electron donor properties of corticotrophic peptides and their biological activity, measurements of donor properties of the peptides should preferably be carried out in aqueous solutions in order to provide the compounds with a natural environment. Therefore, we had to choose a water-soluble acceptor for our experiments.

Most of the well-known acceptors in organic chemistry (*e.g.* chloranil, trinitrobenzenes, picric acid, tetrachlorophthalic acid anhydride *etc.*) are nearly insoluble in water, but pyridinium ions with electron-withdrawing substituents such as *N*-methylnicotinamidium chloride, *N*-methylisonicotinamidium chloride and *N*-methylpicolinamidium chloride[5] combine acceptor properties with high solubility in water.

Other requirements which had to be met by the acceptor were:

a) a high electron affinity,
b) stability over as large a pH range as possible,
c) a large gap between the first and second vacant molecular orbital to avoid complications arising from multiple charge-transfer transitions[6].

An acceptor which fulfils all these requirements very well is *N*,*N*'-dimethyl-4,4'-dipyridylium dichloride (paraquat) as found by Verhoeven[7].

Paraquat (formerly called methylviologen[8]) has been shown to form 1:1 charge-transfer complexes with various anionic and neutral donor species[9-12]; the association constants of such complexes are relatively large[9,10]. The compound is also an inhibitor of photosynthesis[13-15]. The mechanism of this action is not known, but it may involve an electron-transfer process in which the paraquat di-cation is reduced to the free radical mono-cation. The observation[12] that paraquat gives complexes with porphyrin-like molecules is of particular interest in this context.

The almost white paraquat dichloride can be prepared from the red di-iodide with freshly prepared silver chloride in water[7]; *N*,*N*'-dimethyl-4,4'-dipyridylium di-iodide in its turn is easily made from 4,4'-bipyridyl and methyl iodide in dimethylformamide[9].

Experiments in aqueous solutions with indole derivatives as electron donors and various pyridinium chlorides as acceptors revealed that paraquat is a much better electron acceptor than the chlorides of *N*-methylnicotinamide, *N*-methylisonicotinamide and *N*-methylpicolinamide[7]. Therefore, paraquat was used as the electron acceptor in all our experiments.

VI. 3 THE ELECTRON DONORS

VI. 3.1 Introduction

All natural amino acids give coloured charge-transfer complexes[16] with strong electron acceptors (*e.g.* chloranil and tetracyano ethylene). In general, the free amino group is responsible for the formation of so-called n,π-complexes (see ref. 2 p. 6). When the amino group is protected by acylation as in *N*-acetyl- or *N*-*t*-butyloxycarbonyl amino acids, its n-donor properties vanish and only with special amino acids do n- or π-donor properties of the side-chains remain.

Donors are present in the side-chains of the following amino acids: tryptophan (indole), tyrosine (phenol), phenylalanine (benzene), histidine (imidazole), methionine (methyl-thioether), cysteine (thiol) and cystine (dialkyl-disulphide). Tryptophan is by far the strongest donor among these amino acids. It is the only one which gives complexes even with weak electron acceptors.

Since our investigations concern a possible donor function of tryptophan in ACTH, some details will be given about its donor properties and about donor properties of the amino acids which have been substituted for tryptophan[9] in our investigation.

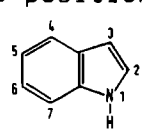
VI. 3.2 Tryptophan

Charge-transfer interaction between indole residues and pyridinium moieties has been studied

intramolecularly in the model compound *N*-(β-indolylethyl)-3-carboxamide pyridinium chloride[17]. From these results it can be concluded that this type of interaction may also occur between tryptophan or tryptophan containing compounds and coenzymes derived from nicotinamide (*e.g.* NAD^+). Indeed, Cilento and Tedeschi[18] observed a charge-transfer band in the spectrum of the glyceraldehyde-3-phosphate dehydrogenase/diphosphopyridine nucleotide complex; it was tentatively ascribed to a charge-transfer interaction between an indole side-chain of the enzyme and the pyridinium ring of DPN by Kosower[19]. In a similar way the indole ring of a single, solvent-exposed tryptophan residue in chicken egg-white lysozyme is considered to be responsible for the yellow-coloured charge-transfer complex with *N*-methylnicotinamidium chloride[20].

Several other investigations with simple model compounds have confirmed the good electron donor properties of indole and indole derivatives, including tryptophan, both in intramolecular[3,21,22] and intermolecular complexes[3,5,7,21,23,24].

Szent Györgyi *et al.* showed that of the four naturally occurring aromatic amino acids, tryptophan is the only one which forms an electron transfer complex with riboflavin[25]. They suggest that the donor site of indoles is localized at the 2-3 position in indole[26].



Calculation of the formal negative charge distribution shows a high density in the 3-position[27].

VI. 3.3 Phenylalanine

Cilento and Tedeschi observed the formation of a weak CT complex between a model compound derived from NAD^+ and phenylalanine[18]. A weak absorption band ascribed to an intramolecular complex was also found for *N*-(β-phenylethyl)-3-carbamoylpyridinium chloride[17]. In both cases the corresponding indolyl compounds appeared to be much better donors.

Hofmann *et al.*, studying substitution of phenylalanine for tryptophan[9] in ACTH analogues, found a nearly complete loss of steroidogenic activity in |Gln^5, Phe^9|-β-corticotrophin-(1-20)-amide and also a lowering of the melanocyte-stimulating activity[28].

If the charge-transfer properties of tryptophan[9] are involved in the biological potency of ACTH peptides, it would be understandable that phenylalanine analogues should have very low activities since phenylalanine seems to be a poor electron donor in comparison with tryptophan[25].

This has been further substantiated in our investigations in which donor properties of several tryptophan-containing peptides, including ACTH-(1-24), have been compared with those of corresponding compounds in which tryptophan was exchanged for phenylalanine.

VI. 3.4 Pentamethylphenylalanine

Since there are no natural amino acids with donor properties comparable to those of tryptophan, replacement of tryptophan residues by equally potent electron donors is only possible with unnatural amino acids.

Schwyzer and coworkers[3,21] found that *N*,*N*-dimethyl-*p*-toluidine and hexamethylbenzene (*i.e.* see [1] and [2]) possess even better donor properties than indole towards acceptors like 4-nitrophthalimide in organic solvents. When, instead of the simple aromatic donors just mentioned, corresponding α-amino-protected amino acid derivatives were complexed with 4-nitrophthalimide or 4-nitrophthaloyl glycine ethyl ester in organic solvents, it appeared that particularly the pentamethylphenylalanine derivative had donor properties comparable to those of tryptophan derivatives (cf. table VI-1).

Table VI-1

Acceptor: 4-nitrophthaloyl glycine ethyl ester
Solvent : ethanol (25°C)

Donor	K(l/mole)	ε_{da}	λ_{max}(nm)
Benzyloxycarbonyl-DL-pentamethylphenylalanine	2.3	500	370
N^{α}-acetyl-L-*p*-dimethylaminophenylalanine	0.54	664	460
N^{α}-acetyl-L-tryptophan	1.56	712	355

N^{α}-Acetyl-L-*p*-dimethylaminophenylalanine has a much lower K-value. Moreover, its introduction into an ACTH-(1-24)-tetracosapeptide can give rise to difficulties in the synthetic procedure as a consequence of the rather basic side-chain. Therefore, we thought it advisable to synthesize and resolve DL-pentamethylphenylalanine and to use this unnatural amino acid in the investigations into a possible relationship between donor properties of the amino acid residue in position 9 and the biological activity of corticotrophic peptides.

VI. 4 COMPARISON OF TRYPTOPHAN CONTAINING PEPTIDES WITH THE CORRESPONDING PENTAMETHYLPHENYLALANINE AND PHENYLALANINE PEPTIDES

VI. 4.1 Introduction

The only peptides from which electron donor properties ascribable to an incorporated tryptophan residue have been measured, are the ACTH-(5-10) fragment, ACTH-(1-24) and the natural hog ACTH with 39 amino acids[30]. The measurements were performed in water and diluted acetic acid with *N*-methylisonicotinamidium chloride as the acceptor.

In view of the scarcity of data about the electron transfer properties of tryptophan containing peptides, our measurements have not been restricted to the tetracosapeptides which have been tested for biological activity, but have also been performed with some intermediates from the

synthesis of the end-products. For these measurements we selected the free ACTH-(7-10) fragments and the dipeptides H-X-Gly-OMe.HCl (X = Trp, Pmp and Phe, respectively). The three variable amino acids at the 9-position were themselves all measured as their *N*-acetyl derivatives in order to obtain corresponding values for comparison within the series of Trp, Pmp and Phe derivatives.

VI. 4.2 Methods

VI. 4.2.1 Performance of the measurements

All spectrophotometric titrations were carried out with a Zeiss PMQ-II spectrophotometer at room temperature in a closed quartz cell of 1 cm (the same cell was used in all determinations).

To the cuvette containing 2.0 ml of a dilute (about $1\text{-}2\times10^{-3}$ molar) solution of the donor in water (except for the *N*-acetyl amino acids which were measured in acetic acid-water (1:4)) increasing amounts of solid *N*,*N*'-dimethyl-4,4'-dipyridylium dichloride (paraquat) were added in 7 steps and the increase in the CT-absorption at various wavelengths was monitored (550-350 nm) after each step. The correction for the concomitant volume expansion was calculated from the formula

$V_y = V_o + y.0.0008^7$, where V_y = volume in ml after addition of y mg of the solid acceptor to V_o ml of solution.

In most cases association constants and extinction coefficients of the peptide complexes were de-

termined using the formula's given in section VI.I. This was not done for the phenylalanine containing compounds, except for [Phe^{9}]-ACTH-(1-24), since the complexes of these substances did not show any CT-absorption. In the figures (VI, 4-7) only the corrected absorption curves for the complexes at the highest concentration of acceptor used are given.

VI. 4.2.2 Influence of the solvent

An aqueous solution with the appropriate donor-concentration (about 10^{-3} molar) could not be obtained with *N*-acetyl-pentamethylphenylalanine in contradistinction to *N*-acetyl-tryptophan, *N*-acetyl-phenylalanine and the various peptides. Adequate concentrations for all compounds could be obtained, however, with solvent mixtures such as acetic acid-water or acetic acid-dilute hydrochloric acid.

The solvent influence on the stability of the complex between *N*-acetyl-tryptophan and *N*-methyl-nicotinamidium chloride has been studied by Deranleau and Schwyzer[5] in ethanol-water mixtures. They found that an increase in the ethanol concentration markedly decreased the association constant of the complex, without affecting the extinction coefficient appreciably.

We investigated a possible solvent influence in acetic acid-water mixtures for the very strong electron donor 3-methylindole (skatole) in 20% acetic acid, 80% acetic acid and acetic acid-0.1 N hydrochloric acid (4:1, ^{v}/v) using the same end-concentration of the components. Qualitative ex-

periments indicated that in this case the intensity of the CT-bands was considerably higher when water or 20% acetic acid were used as the solvent whereas the position of the bands was nearly the same in all solvent mixtures employed (fig. VI, 2). Probably, water stabilizes the complexes more than acetic acid. The influence of these solvent systems on the absorption of paraquat differs only below 375 nm, as is shown in fig. VI, 3.

In order to restrict the consequences of the solvent influence on K and ε the association constants and the extinction coefficients for the complexes formed between paraquat and *N*-acetyl-tryptophan and *N*-acetyl-pentamethylphenylalanine were determined in 20% acetic acid as the solvent. In all other measurements, water was used as the solvent.

VI. 4.2.3 Synthesis of the compounds

N-Acetyl-L-tryptophan and *N*-acetyl-L-phenylalanine were prepared from the amino acids by treatment with acetic anhydride in aqueous sodium hydroxide solution according to the method of du Vigneaud[29]. The synthesis of *N*-acetyl-L-pentamethylphenylalanine has been described in chapter II. In chapter III and IV the synthesis of the pentamethylphenylalanine and phenylalanine peptides, respectively, have been described. Details of the dipeptide H-Trp-Gly-OMe.HCl, with which only qualitative experiments were carried out since it should have about the same electron donor prop-

erties as the previously examined H-Trp-OMe.HCl[7], are given in chapter VIII.

VI. 4.3 Experimental Results

In tabel VI-2 the donor and acceptor concentrations, after volume correction, in the beginning and at the end of the experiment are given. Table VI-3 summarizes the results obtained from the complexing of paraquat with the various donors. Since there was no measurable CT-absorption from complexes with phenylalanine containing compounds, no determinations of K and ε_{da} could be carried out, except for the tetracosapeptide.

The corrected absorption curves for the complexes at the highest concentration of acceptor used are given in figures VI, 4, 5, 6 and 7.

Table VI-2

Donor	Donor concentration (Molar, x 10^{-3})		Acceptor concentration (Molar, x 10^{-2})	
	beginning (no paraquat)	end	beginning	end
N-acetyl-L-Trp-OH	1.33	1.19	15.60	53.71
N-acetyl-L-Pmp-OH	0.85	0.76	15.62	53.77
N-acetyl-L-Phe-OH	1.76	1.59	-	46.80
H-Trp-Gly-OMe.HCl	1.90	1.72	-	44.34
H-Pmp-Gly-OMe.HCl	1.73	1.54	19.10	51.00
H-Phe-Gly-OMe.HCl	1.85	1.65	-	52.13
H-Phe-Arg-Trp-Gly-OH.HCl.$2H_2O$	1.09	0.97	15.66	53.11
H-Phe-Arg-Pmp-Gly-OH.HCl.$2H_2O$	1.14	1.01	15.62	55.59
H-Phe-Arg-Phe-Gly-OH.HCl.H_2O	2.34	2.08	-	53.32
[Trp^9]-ACTH-(1-24) (peptide content 80.6%)	0.94	0.83	16.02	51.64
[Pmp^9]-ACTH-(1-24) (peptide content 75%)	0.82	0.72	16.56	51.93
[Phe^9]-ACTH-(1-24) (peptide content 58%)	0.96	0.85	16.31	52.40

Table VI-3

Calculated data from the measurements of the charge-transfer complexes of paraquat with the *N*-acetyl amino acids in 20% acetic acid and with various peptides in water, at room temperature.

Compound	λ(nm)	K (l/mole)	K.ε	$K.\varepsilon/\overline{K}$
N-acetyl-Trp-OH	360	2.04	1596	694
	370	2.14	1772	770
	380	2.21	1875	815
	390	2.34	1950	847
	410	2.56	1955	850
N-acetyl-Pmp-OH	360	0.17	456	1520
	370	0.33	527	1750
	380	0.25	521	1737
	390	0.26	510	1700
	410	0.38	458	1527
H-Trp-OMe.HCl[7]	370	2.32	1840	680
	400	2.88	2003	740
	420	2.64	1872	690
	450	2.86	1522	564
H-Pmp-Gly-OMe.HCl	360	1.46	1400	1052
	370	1.43	1422	1069
	380	1.29	1355	1019
	390	1.23	1280	962
	410	1.26	1058	795

Table VI-3, continued

Compound	λ(nm)	K (1/mole)	K.ε	K.ε/$\overline{K}$
H-Phe-Arg-Trp-Gly-OH.HCl	360	3.59	2604	620
	370	3.77	2820	671
	380	4.14	3075	732
	390	4.43	4631	1103
	410	4.95	3463	825
H-Phe-Arg-Pmp-Gly-OH.HCl	360	1.56	1235	772
	370	1.46	1288	805
	380	1.62	1368	855
	390	1.65	1369	856
	410	1.64	1211	757
[Trp^{9}]-ACTH-(1-24)	360	1.46	2528	1397
	370	1.55	2732	1509
	380	1.81	2776	1534
	390	1.93	2771	1531
	400	1.93	2634	1455
	410	2.21	2566	1418
[Pmp^{9}]-ACTH-(1-24)	360	1.93	2813	1655
	370	1.74	2763	1625
	380	1.79	2701	1590
	390	1.55	2475	1456
	400	1.53	2258	1328
	410	1.68	2096	1233
[Phe^{9}]-ACTH-(1-24)	360	0.26	880	2444
	370	0.23	744	2067
	380	0.26	613	1703
	390	0.30	495	1375
	400	0.56	359	997
	410	0.58	276	767

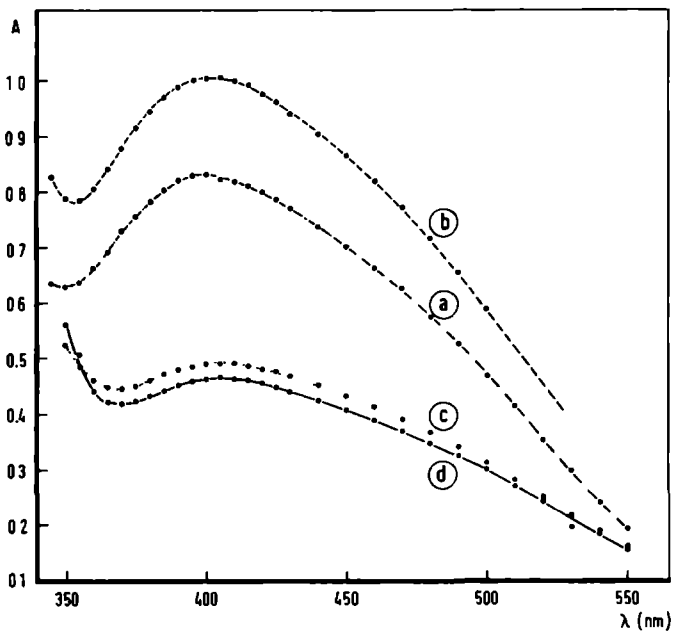


Fig. VI, 2 - Absorption spectrum of the CT-complex formed between skatole and paraquat (concentrations 1.4×10^{-3} M and 41.4×10^{-2} M, respectively) as measured in

(a) H_2O
(b) HOAc-H_2O (1:4, $^{v}/v$)
(c) HOAc-H_2O (4:1, $^{v}/v$)
(d) HOAc-0.1 N HCl (4:1, $^{v}/v$)

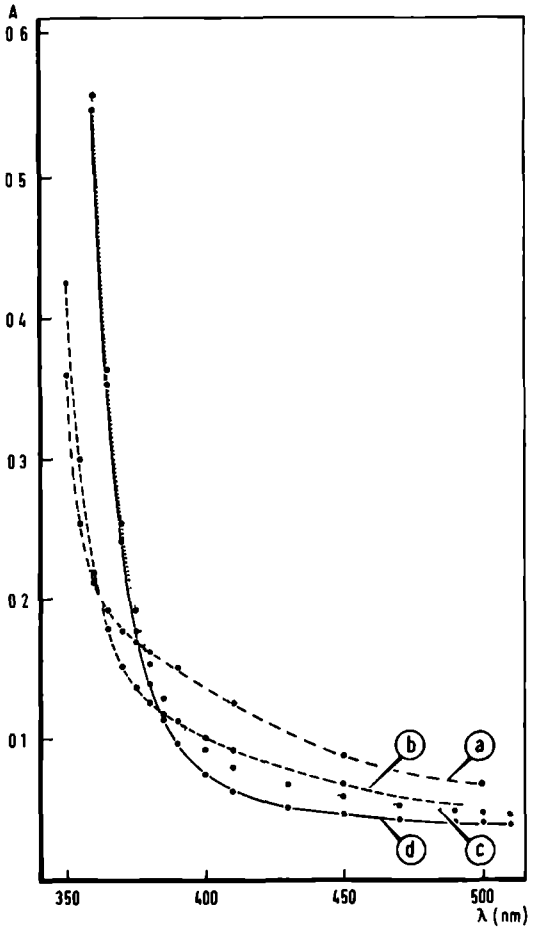


Fig. VI, 3 - Influence of the solvent systems (a) (b) (c) and (d) (see fig. VI, 2) on the paraquat absorption (concentration about 115 mg/ml).

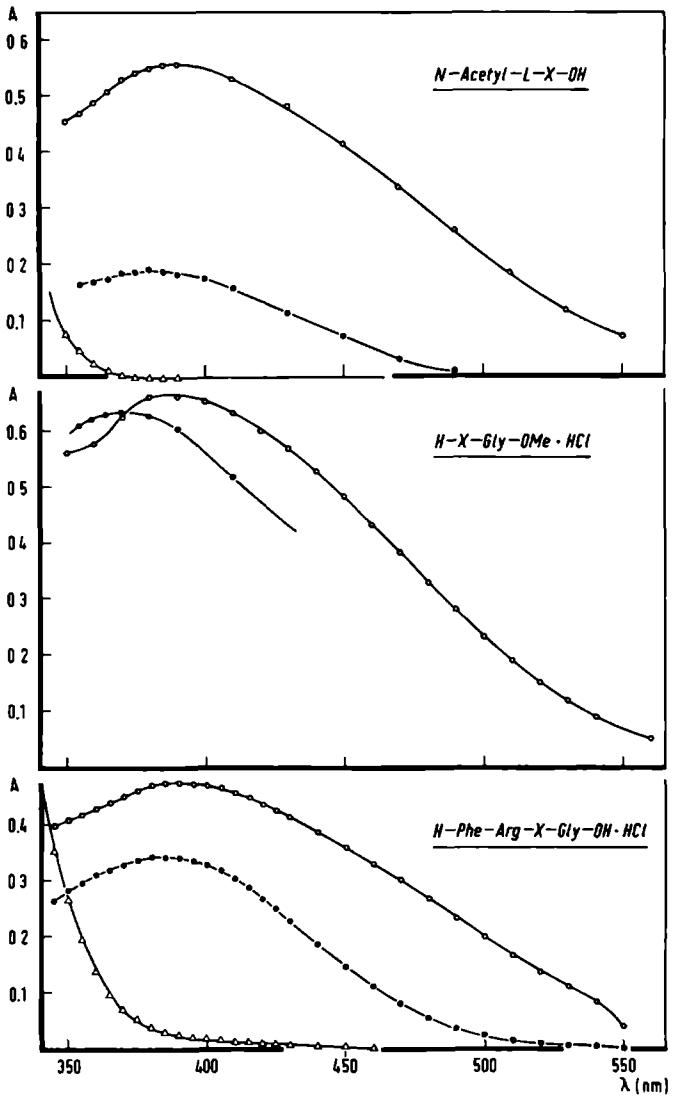


Fig. VI, 4-6 - Corrected absorption curves for the CT-complexes of several compounds and paraquat at the highest paraquat concentration, measured in 20% HOAc (fig. 4) or H_2O at room temperature. —o—o— X = Trp, —●—●— X = Pmp, —Δ—Δ— X = Phe.

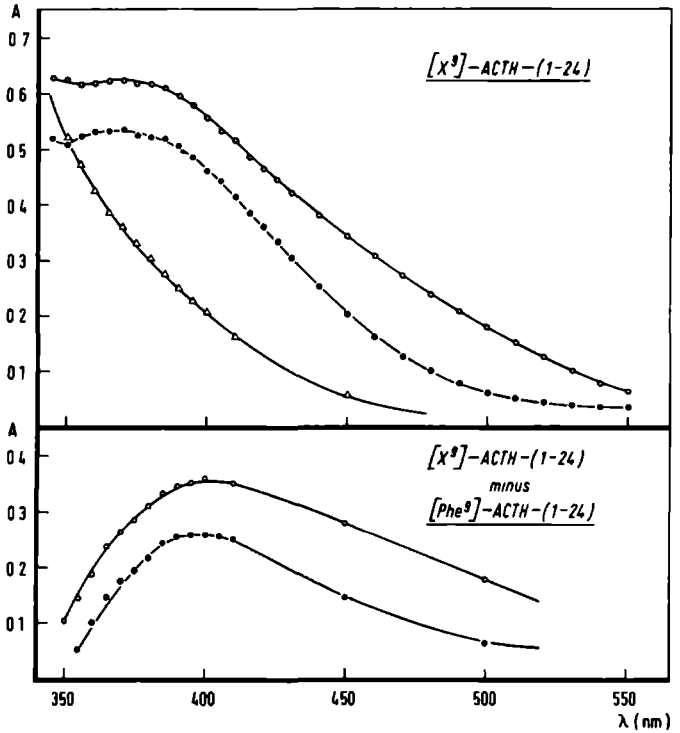


Fig. VI, 7 (top) - Corrected absorption curves for the CT-complexes of $[X^9]$-ACTH-(1-24) and paraquat at the highest paraquat concentration, measured in water at room temperature. —o—o— X = Trp, —●—●— X = Pmp, —Δ—Δ— X = Phe.

Fig. VI, 8 (bottom) - Absorption curves obtained after subtracting curve —Δ—Δ— from curves —o—o— and —●—●— in fig. VI, 7. —o—o— X = Trp, —●—●— X = Pmp.

VI. 4.4 Discussion

In table VI-4 the complex constants for several tryptophan- and pentamethylphenylalanine containing compounds are summarized.

CT-absorption for the simple tryptophan containing peptides takes place at a slightly longer wavelength than for the corresponding pentamethylphenylalanine peptides, while the small phenylalanine peptides do not show any CT-absorption at all. The association constants for the small tryptophan peptides are markedly larger than for the pentamethylphenylalanine peptides while the ε^*_{max} values are in general somewhat lower.

The results found for *N*-acetyl-Trp-OH in 20% acetic acid agree with the solvent dependence of the complex constants as was found by Deranleau and Schwyzer[5] in ethanol-water mixtures. The low maximum saturation attained in the *N*-acetyl-L-Pmp-OH/paraquat complex indicates that the values found for K and ε have a larger deviation than in the other cases.

In the dipeptide, the positive charge of the $-\overset{+}{N}H_3$ group may be responsible for the less efficient complexing of paraquat with the side-chain (the same was suggested for H-Trp-OMe.HCl by Verhoeven[7]).

With the tetrapeptides, where the donor amino acids have no free α-amino group, complexing in the case of Trp and Pmp is much better (yellow-

Table VI-4

Characteristic parameters for the charge-transfer complexes of *N*-methylnicotinamidium chloride (A), paraquat (B) and *N*-methylisonicotinamidium chloride (C) with several tryptophan- and pentamethylphenylalanine containing compounds.

Complex	Solvent	λ_{max} (nm)	$\overline{K}$ (l/mole)	ε^*_{max}	S_{max} (%)	Ref.
N-acetyl-L-Trp-OH/A	H_2O	-	5.2	830	-	5
" /A	20% EtOH	-	3.6	800	-	5
" /B	20% HOAc	387±3	2.3 ±0.2	820±70	55	this work
N-acetyl-L-Pmp-OH/B	"	380±2	0.30±0.05	1790±300	14	" "
H-Trp-OMe.HCl/B	H_2O	383±2	2.7 ±0.2	740±50	56	7
H-Pmp-Gly-OMe.HCl/B	"	370±5	1.33±0.1	1074±80	37	this work
H-Phe-Arg-Trp-Gly-OH.HCl/B	"	390±5	4.2 ±0.5	734±85	69	" "
H-Phe-Arg-Pmp-Gly-OH.HCl/B	"	385±5	1.6 ±0.04	858±21	47	" "
H-Glu-His-Phe-Arg-Trp-Gly-OH/C	"	365	3.30	445	-	30
[Trp^9]-ACTH-(1-24)/C	0.01N HOAc	-	~1.8	~732	-	30
" /C	0.2M $AcO^-NH_y^+$	~365	2.00	779	-	30
" /C	H_2O	-	~1.6	~825	-	30
" /B	"	370±2	1.8 ±0.3	1560±250	48	this work
[Pmp^9]-ACTH-(1-24)/B	"	370±2	1.7 ±0.2	1630±200	47	" "
[Phe^9]-ACTH-(1-24)/B	"	no max.	0.36±0.13	-	16	" "
hog ACTH-(1-39)/C	0.01N HOAc	~365	1.9	480	-	30

orange colour). Again, the phenylalanine tetrapeptide does not show any CT-absorption.

For the yellow-coloured [Trp^{9}]-ACTH-(1-24)/paraquat complex, the value for K decreases from about 4.2 (tetrapeptide) to about 1.8, while on the other hand the association constant for the yellow [Pmp^{9}]-ACTH-(1-24)/paraquat complex does not change (about 1.7). A possible explanation is that the indole moiety, because of the lower symmetry and strong localization of its highest occupied molecular orbital, makes more specific demands for complexing with paraquat than a pentamethylphenyl residue with a higher symmetrical and more delocalized high occupied molecular orbital.

Complexing of [Phe^{9}]-ACTH-(1-24) with paraquat results in a very slightly yellow-coloured solution. From fig. VI, 7 it can be seen that there is no maximum in the curve.

To eliminate possible influences of all the other amino acids in the tetracosapeptides on complex formation, especially those of the tyrosine residues in positions 2 and 23 which show reasonable CT-absorption[7], curve 3 in fig. VI, 7 can be subtracted from curves 1 and 2. The resulting curves (fig. VI, 8) have λ_{max} at about 400 nm which is about the same value as found with the very good donor skatole (see ref. 7 and fig. VI, 2).

We conclude that, if the donor properties of tryptophan in ACTH are involved in the biological activity, the pentamethylphenylalanine analogue must exhibit a nearly equal potency, whereas the phenylalanine peptide should be almost inactive.

VI. 5 SYNTHETIC PART

N,N'-Dimethyl-4,4'-dipyridylium dichloride

100 mmoles (15.6 g) of 4,4'-bipyridyl (Fluka) were dissolved in 250 ml of pure DMF and 250 mmoles (35.4 g) of methyliodide were added. The reaction flask was kept in the dark for 24 h at room temperature. Then, about 1.5 l of ether were added with stirring and the red *N,N'*-dimethyl-4,4'-dipyridylium di-iodide was filtered and washed with ether (96%). To a suspension of this compound in water, an excess (about 4 equivalents) of freshly prepared AgCl (from $AgNO_3$ and HCl) was added with stirring. After a few hours the AgI formed and the excess of AgCl were removed by filtration and the filtrate was evaporated. After drying, the residue was dissolved in ethanol and ether was added with stirring. The almost white paraquat was obtained in about 98% yield; m.p.: >330°C.

Analysis:

$C_{12}H_{14}N_2Cl_2$	Calcd.:	% C	56.05	% H	5.49	% N	10.89	% Cl	27.57
(257.16)	Found :	% C	55.6	% H	5.7	% N	10.7	% Cl	27.5

Although it is only slightly hygroscopic it is advisable to store the product in a desiccator.

N-Acetyl-L-tryptophan

The acetylation of tryptophan was carried out with acetic acid anhydride in NaOH[29]. The product was recrystallized from water.

Yield	: 78%	Lit.[29]	-
M.p.	: 175-177°C		189-190°C
$[\alpha]_D^{21}$	: +25.1° (c = 1.0, MeOH) (Perkin-Elmer 241)		+30° (1 eq. NaOH)
TS	: Rf = 0.60 (B) (UV and R-H)		

Analysis:

$C_{13}H_{14}N_2O_3$	Calcd.:	% C 63.40	% H 5.73	% N 11.38
(246.27)	Found :	% C 63.1	% H 5.9	% N 11.4

N-Acetyl-L-phenylalanine

The same method was used to obtain this compound, which was also recrystallized from water.

Yield	: 70%	Lit.[29] -	[31] -
M.p.	: 165-166.5°C	172°	171-172°
$[\alpha]_D^{21}$	: +45.3° (c = 1.0, EtOH) (Perkin-Elmer 241)	+51.4° (c = 1, EtOH)	+47.5° (c = 4, EtOH)
TS	: Rf = 0.60 (B) (R-H)		

Analysis:

$C_{11}H_{13}NO_3$	Calcd.:	% C 63.76	% H 6.32	% N 6.76
(207.23)	Found :	% C 63.7	% H 6.3	% N 6.8

REFERENCES

1. Foster, R. (1969) Organic Charge Transfer Complexes, Academic Press, London.
2. Briegleb, C. (1961) "Elektronen-Donator-Acceptor-Komplexe", Springer-Verlag, Berlin.
3. Carrión, J.P., Donzel, B., Deranleau, D.A., Esko, K., Moser, P. and Schwyzer, R. (1967) in Peptides 1966, Proceedings of the 8th European Peptide Symposium (Beyerman, H.C., van de Linde, A. and Maassen van den Brink, W., eds.) pp. 177-188, North-Holland Publishing Co., Amsterdam.
4. Foster, R., Hammick, D.L. and Wardley, A.A. (1953) *J. Chem. Soc.* 3817-3820.
5. Deranleau, D.A. and Schwyzer, R. (1969) *Biochemistry* 9, 126-134.
6. Verhoeven, J.W., Dirkx, I.P. and de Boer, Th.J. (1969) *Tetrahedron* 25, 3395-3405.

7. Verhoeven, J.W. and Schwyzer, R., to be published.
8. Michaelis, L. (1935) *Chem. Rev.* 16, 243-286.
9. Nakahara, A. and Wang, J.H. (1963) *J. Phys. Chem.* 67, 496-498.
10. Ledwith, A. and Iles, D.H. (1968) *Chem. in Britain* 4, 266-267.
11. Woods, J.H. (1967) Thesis, Liverpool.
12. Macfarlane, A.J. (1968) Thesis, Oxford.
13. Jagendorf, A.T. and Avron, M. (1958) *J. Biol. Chem.* 231, 277-290.
14. Hill, R. and Walker, D.A. (1959) *Plant Physiol.* 34, 240-245.
15. Boon, W.R. (1965) *Chem. & Ind.* 782-788.
16. Slifkin, M.A. (1964) *Spectrochim. Acta* 20, 1543-1554 and earlier papers.
17. Shifrin, S. (1964) *Biochim. Biophys. Acta* 81, 205-213.
18. Cilento, G. and Tedeschi, P. (1961) *J. Biol. Chem.* 236, 907-910.
19. Kosower, E.M. (1956) *J. Amer. Chem. Soc.* 78, 3497-3501.
20. Deranleau, D.A., Bradshaw, R.A. and Schwyzer, R. (1969) *Proc. Natl. Acad. Sci.* USA 63, 885-889.
21. Carrión, J.P., Deranleau, D.A., Donzel, B., Esko, K., Moser, P. and Schwyzer, R. (1968) *Helv. Chim. Acta* 51, 459-481.
22. Matsunaga, Y. (1973) *Bull. Chem. Soc. Japan* 46, 998-999.
23. Shifrin, S. (1964) *Biochemistry* 3, 829-833.
24. Foster, R. and Fyfe, C.A. (1966) *J. Chem. Soc.* (B), 926-929.
25. Isenberg, I. and Szent-Györgyi, A. (1958) *Proc. Natl. Acad. Sci.* USA 44, 857-862.
26. Szent-Györgyi, A. and Isenberg, I. (1969) *Proc. Natl. Acad. Sci.* USA 46, 1334-1336.

Szent-Györgyi, A., Isenberg, I. and McLaughlin, J. (1961) *Proc. Natl. Acad. Sci.* USA, 47, 1089-1093.
Smaller, B., Isenberg, I. and Baird Jr., S.L. (1961) *Nature* 191, 168-169.

27. Pullman, B. and Pullman, A. (1952) Les théories electroniques de la chimie organique, Masson, Paris.
28. Hofmann, K., Andreatta, R., Bohn, H. and Moroder, L. (1970) *J. Med. Chem.* 13, 339-345.
29. Du Vigneaud, V. and Sealock, R.R. (1932) *J. Biol. Chem.* 96, 511-517.
30. Schwyzer, R., Schiller, P., Fauchère, J.-L., Karlaganis, G. and Pelican, G.-M. (1971) in Structure-activity relationships of protein and polypeptide hormones, Proceedings of the 2nd Intern. Symp., Liège (Margoulies, M. and Greenwood, F.C., eds.) pp. 167-175, Excerpta Medica Foundation, Amsterdam.
31. Overby, L.R. and Ingersoll, A.W. (1951) *J. Amer. Chem. Soc.* 73, 3363-3366.

CHAPTER VII

BIOLOGICAL ACTIVITIES OF THE FOUR ACTH AND TWO α-MSH ANALOGUES

VII. 1 INTRODUCTION

As already mentioned in chapter I, stimulation of the adrenal cortex to produce and release steroid hormones and lipolysis in fat cells are the main activities of ACTH.

In structure-function studies measurement of steroidogenesis can be carried out *in vivo*[1-5] and, in the method described by Saffran and Schally[6] or modifications of this[7], also *in vitro*. Rats are preferably used for the determination of the steroidogenic potency of ACTH analogues, since corticosterone is the main steroid secreted by rat adrenals. Steroid determinations are confined to measurements of corticosterone using the acid fluorescence technique of Silber *et al.*[8] as modified by Guillemin *et al.*[4,9].

For the *in vitro* determination of the extra-adrenal activity of ACTH in fat cells, the methods of Jungas and Ball[10], and of Rodbell[11] are used. The unesterified fatty acids released into the incubation medium are measured by titration[12] and the glycerol content is determined according to the method of Wieland[13] or the method of Korn[14]. Lipolytic activity *in vivo* can be estimated on the

basis of the concentration of unesterified fatty acids determined colorimetrically in the plasma as described by Duncombe[15].

Another important extra-adrenal activity of ACTH is the stimulation of the melanocytes. This skin-darkening effect has mainly been investigated with frog skin in the *in vitro* test of Shizume *et al.*[16] or in the procedure of Schuler *et al.*[7]. Lizards have also proved to be very good test animals for the *in vitro* examination of the melanocyte-expanding activity of α-MSH and ACTH peptides [17,18]. The *in vivo* method of Kastin and Ross[19] has not found the same wide application as the *in vitro* methods.

VII. 2 MATERIALS AND METHODS

Peptides. The compounds that have been tested for various biological activities are [Pmp^9]-ACTH-(1-24) and its D-Ser^1 analogue (see chapter III), [Phe^9]-ACTH-(1-24) and its D-Ser^1 analogue (chapter IV), and the two α-MSH analogues, *viz.* [Pmp^9]- and [Phe^9]-α-MSH, described in chapter V.

Steroidogenesis. In vitro, adrenal slices were incubated for 2 hours according to the method described by Saffran and Schally[6]. The corticosterone content of the incubation medium was measured by acid fluorescence[9]. For *in vivo* experiments, rats (about 100 g) which had been hypophysectomized about 24 hours previously, were used.

Lipolysis. Lipolytic activity *in vitro* was determined essentially as described by Jungas and Ball [10]. After 2 hours of incubation the unesterified fatty acids were measured by the method of Dole [12]. *In vivo* experiments were conducted according to the technique of Duncombe [15].

Melanocyte-stimulating activity. The tests were performed *in vitro* with the skin of *Anolis Carolinensis* [18]. Some of the peptides were also tested with frog skin [7].

VII. 3 RESULTS

Steroidogenesis in vitro

The pentamethylphenylalanine[9] ACTH analogues had to be tested in doses up to 100 γ/ml since there was no noticeable corticosterone release at doses lower than 10 γ/ml. Compared with normal ACTH-(1-24), the activity of [Pmp[9]]-ACTH-(1-24) is about $^{1}/_{600}$, while [D-Ser[1], Pmp[9]]-ACTH-(1-24) is only slightly more active. ([D-Ser[1]]-ACTH-(1-24) is about twice as active as its L-Ser[1] analogue).

The activity of [Phe[9]]-ACTH-(1-24) is about 1% of that of the normal peptide, while [D-Ser[1], Phe[9]]-ACTH-(1-24) has an activity of about $^{1}/_{66}$ of the normal ACTH-(1-24).

The *in vivo* determination of the Phe[9] peptides show a reasonable high but short steroidogenic response when compared with the normal ACTH-(1-24) (dose: 1 mg/kg body weight).

Lipolysis

In *in vitro* experiments with rats no activity at all was found when 10-100 γ/ml doses of the Pmp peptides were used. The same experiments with ACTH-(1-24) revealed full activity with doses of 0.01-0.1 γ/ml.

In *in vivo* experiments with rats, the Pmp^9-analogues showed no lipolytic activity in doses up to 300 γ/kg. The same was found when rabbits were used as test animals (doses up to 10 γ/kg). The rabbit experiments were done because the effect of the *o*-nitrophenylsulphenyl derivative of ACTH on lipolysis in rabbits is quite different from the effect on lipolysis in rats[20].

Melanophore-expanding properties in vitro

Contrary to the very low steroidogenesis and lipolysis found, the melanophore-expanding activities of the [Pmp^9]-ACTH-(1-24) peptides were surprisingly high. Table VII-1 summarizes the results obtained in two tests, on lizard and frog skin, respectively; the activity of α-MSH was arbitrarily set at 1 and the values found were corrected for variations in the peptide content of the samples.

Table VII-1

Compound	Activity		Peptide content (%)*	Corrected values	
	lizards	frogs		lizards	frogs
[Trp^{9}]-ACTH-(1-24)	∿0.05	-	∿80	0.052	-
[Pmp^{9}]-ACTH-(1-24)	0.01 -0.02	0.01-0.03	∿75	0.011-0.022	0.011-0.034
[D-Ser^{1}, Pmp^{9}]-ACTH-(1-24)	0.04 -0.1	∿0.05	∿75	0.045-0.11	∿0.056
[Phe^{9}]-ACTH-(1-24)	0.004-0.01	-	∿58	0.006-0.015	-
[D-Ser^{1}, Phe^{9}]-ACTH-(1-24)	0.014-0.05	-	∿58	0.021-0.075	-
[Pmp^{9}]-α-MSH	0.25 -0.6	}0.1-0.3	∿73	0.29 -0.69	}0.12-0.36
[Phe^{9}]-α-MSH	-		∿70	-	

* The peptide content of the standard was 84%.

VII. 4 DISCUSSION

The results reveal that the steroidogenic and lipolytic activity of the two [Pmp^{9}]-ACTH peptides on the one hand and the melanocyte-expanding activity on the other, contrast markedly. While with [Pmp^{9}]-ACTH-(1-24) an almost negligible corticosteroid production, no mobilization of unesterified fatty acids, and no release of glycerol are found, the melanocyte-expanding activity tested with lizard as well as frog skin, is 20-50% of that of normal ACTH-(1-24).
The influence of the introduction of a D-Ser residue in position 1 on steroidogenesis and lipolysis is hardly noticeable but the melanocyte-expanding activity increases about fourfold.

The observed steroidogenic potency of [Phe^{9}-ACTH-(1-24)] (about 1%) agrees very well with the data of Hofmann *et al.*[21] who reported about 2% activity for [Gln^{5}, Phe^{9}]-β-corticotrophin-(1-20)-amide. The increase in steroidogenesis after the introduction of a D-Ser residue in position 1 is somewhat lower than the twofold increase caused by the same substitution in the corresponding tryptophan peptide[22]. The melanocyte-expanding activity of [Phe^{9}]-ACTH-(1-24) is about 15% of the activity of the Trp^{9} peptide; this is slightly more than that reported by Hofmann for the eicosapeptide. The activity is increased by a factor of 4 to 5 in the case of [D-Ser^{1}, Phe^{9}]-ACTH-(1-24).

Whereas our data on melanocyte-expanding activities of the tetracosapeptide suggest that [Pmp^9]-peptides are significantly more active than the corresponding [Phe^9]-peptides, such a difference could not be clearly established between [Pmp^9]- and [Phe^9]-α-MSH. Both compounds have a relative activity between 0.1 and 0.3. The former compound might be more active on lizard skin than on frog skin.

It is clear that our results cannot give a definite answer to the question as to whether adequate electron donor properties of the amino acid residue in position 9 of corticotrophic compounds are an essential factor in the determination of biological activity. As far as steroidogenesis and lipolysis are concerned it is certainly not the only decisive factor, but it remains uncertain whether charge-transfer interaction between this distinct residue and the corresponding receptor are at all concerned in the biological activity of this type of compound. The comparable donor properties of Trp and Pmp are attended by rather large structural differences, and these differences may cause variations in other parameters (which may be important for biological activity) on substiution of Pmp^9 for Trp^9 in ACTH analogues. For this reason further investigations on analogues having residues structurally more related to tryptophan, *e.g.* β-(3-benzo[b]thienyl)-alanine, β-(benzofuranyl)-alanine or N^i-methyl-tryptophan at position 9 will be of importance.

The remarkable melanocyte-stimulating activity of the Pmp - analogues is an interesting result. According to Hofmann[23] up to now all alterations in the essential part of ACTH-analogues that have lowered adrenocorticotrophic activity have also brought about a proportional lowering in melanocyte-stimulating activity. With our Pmp-derivatives a separation between both types of activity could be realized for the first time.

In the melanocyte stimulation, charge-transfer interaction between the side-chain of the amino acid residue in position 9 is compatible with the working hypothesis when the analogues are tested with lizard skin. The comparable activities of [Pmp^{9}]- and [Phe^{9}]-α-MSH in the frog skin test however, do not substantiate this result. Future investigations must therefore allow for a discrimination between an also possible hydrophobic interaction and the anticipated charge-transfer interaction in the excitation of the MSH receptor.

From the beginning of our investigations we have realized that studies on the special function of a tryptophan residue, occurring in the essential part of a peptide hormone, could also be conducted with analogues of endocrine factors other than ACTH. From cooperation with other research groups during our investigation, some results became available concerning the effect of substitutions of Pmp for Trp in LH-RH and a gastrin tetrapeptide. Because some of these results seem likely to stimulate the continuation

of such investigations, they will be presented in the following section.

VII. 5 OTHER HORMONAL PEPTIDES WITH L-TRYPTOPHAN REPLACED BY L-PENTAMETHYLPHENYLALANINE

VII. 5.1 Luteinizing hormone-releasing hormone (LH-RH)

The hypothalamus regulates the release of the luteinizing hormone (LH) and the follicle-stimulating hormone (FSH) from the anterior pituitary gland by means of neurohumoral factors designated as LH-releasing hormone (LH-RH) and FSH-releasing hormone (FSH-RH)[24]. The isolation of a polypeptide from porcine hypothalamic extracts which has both LH-RH and FSH-RH activity, and the finding that FSH-RH activity cannot be separated from LH-RH was reported by Schally and coworkers[25,26]; they found the hormone to be a decapeptide amide, containing one tryptophan residue at position 3: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2[27]. The synthesis of this decapeptide has recently been described[28].

From further investigations of Schally's group it was found that the tryptophan residue is essential for biological activity[29]. Replacement of tryptophan by amino acids containing simple hydrocarbon side-chains leads to analogues having virtually no LH-releasing activity[30]. Replacement by phenylalanine[30] or tyrosine[31], however, results in compounds showing significant, though very low,

levels of LH-RH activity.

Further investigations concerning the role of tryptophan[3] have been carried out in a joint investigation with Coy *et al.*[32] in which successive substitutions in position 3 were conducted using: pentamethylphenylalanine, 5-fluorotryptophan, *p*-aminophenylalanine, *p*-nitrophenylalanine and histidine. In ovariectomized rats pretreated with oestrogen and progesterone[33] the LH-RH analogues were found to have LH-releasing activities of 69%, 6%, 0.6%, 0.01% and *ca.* 0.05%, respectively, when compared to LH-RH itself. LH- and FSH-activities of [Pmp^3]-LH-RH were also determined by an assay based on a 4-hour infusion[34] of the peptide into immature male rats. In this test the LH-releasing potency is 41% and the FSH-releasing potency 34%. Since the only observable similarity in the properties of tryptophan and pentamethylphenylalanine, apart from aromaticity, is their ability to donate π-electrons, it is probable that similar electronic interactions between tryptophan, or pentamethylphenylalanine, and a suitably oriented electron deficient moiety on the pituitary receptor, represents one of the principal criteria for the maintenance of biological function in LH-RH peptides; this might occur either by ensuring binding of the hormone to the receptor, participation in the mechanism promoting LH release, or a combination of both factors.

Introduction of 5-fluorotryptophan, which is a poorer donor than tryptophan but structurally and chemically much more closely related than

pentamethylphenylalanine, causes a considerable reduction in LH-releasing activity. [*p*-NO_2-Phe^3]-LH-RH had diminished activity as compared to [Phe^3]-LH-RH whereas [*p*-NH_2-Phe^3]-LH-RH showed a slightly higher potency than the latter compound. With these peptides it is more difficult to attribute changes in biological activity to the electronic effects of ring substituents since other factors such as size, acid-base behaviour and hydrophobicity might have overriding effects. However, the *p*-NO_2-Phe analogue, containing the strongly electron-withdrawing nitro group, is by far the least active of the phenylalanine analogues.

VII. 5.2 Gastrin tetrapeptide

Gastrin is the generic name for certain peptide hormones which can be extracted from the antral region of the stomach; they are remarkably potent stimulators of gastric secretion, and have a wide range of other activities in the alimentary tract. These were thoroughly investigated by Gregory and Tracy[35], who isolated two pure, closely related compounds from antral mucosa of hogs. Degradative studies[36] indicated that both porcine hormones are heptadecapeptide amides having the same amino acid sequence: pGlu-Gly-Pro-Trp-Met-$(Glu)_5$-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2. Gastrin II differs from gastrin I by the presence of a phenolic sulphate ester group. Investigations into gastrins from other species showed some minor

variations in the amino acid sequence 5-10[37]. Syntheses of porcine[38] and human[39] gastrin have been described.

It has been shown that the *C*-terminal tetrapeptide amide, H-Trp-Met-Asp-Phe-NH_2, is required and is sufficient to elicit the whole range of physiological actions on gastric and pancreatic gland-cells and on gastrointestinal musculature displayed by the natural hormone[40,41].

Structure-function studies on the active tetrapeptide by Morley *et al.*[41,42] showed that substitution of the tryptophan residue by phenylalanine or histidine lowers activity considerably, but that substitution by des-amino tryptophan or des-amino homotryptophan and also introduction of a methyl group at positions 4, 5 or 6, or a hydroxyl group at positions 2 or 5 of the indolyl group of tryptophan, results in highly active compounds. It was concluded[42] that tryptophan is concerned with binding at the site of action.

A possible role for the electron donor properties of the indole moiety in the binding process was investigated by replacement of the tryptophan residue by L-pentamethylphenylalanine. For this purpose the 2,4,5-trichlorophenyl ester of *t*-butyloxycarbonyl-L-pentamethylphenylalanine was prepared according to the method of Pless and Boissonnas[43]. The active ester (see table VII-2) was coupled with H-Met-Asp(OBu^t)-Phe-NH_2.HCl*[44]

* Gift from Dr. J.S. Morley (I.C.I., England)

Table VII-2

	Boc-Pmp-OTcp	Boc-Pmp-Met-Asp(OBu^t)-Phe-NH_2	H-Pmp-Met-Asp-Phe-NH_2.HCl
Yield (%)	60.0	72.1	79.7
M.p. (^oC)	162-163	205.5-206	222-223
$[\alpha]_D^{21}$ (c = 1.0, DMF)	-14.6^o	-32.6^o	-3.3^o
Analysis (theoretical	$C_{25}H_{30}NO_4Cl_3$	$C_{41}H_{61}N_5O_8S.2H_2O$	$C_{32}H_{46}N_5O_6SCl.H_2O$
values in parentheses)	(514.88)	(820.06)	(682.29)
% C	58.1 (58.32)	60.2 (60.05)	55.7 (56.33)
% H	5.9 (5.87)	7.4 (7.99)	6.9 (7.09)
% N	2.6 (2.72)	8.6 (8.54)	10.2 (10.27)
% S		3.8 (3.91)	4.3 (4.70)
% Cl	20.6 (20.66)		5.9 (5.20)

according to the method of Davey *et al.*[44] and the resulting product, after recrystallization from ethanol and drying, deprotected with hydrogen chloride in acetic acid. Some chemical and physical data for the intermediates and the end-product of the synthesis are given in table VII-2.

The tetrapeptide has been assayed in two dogs*. Doses of 1 and 2 µg/kg/hr were given by intravenous infusion in the absence of background stimulation. Gastric secretion was collected at 15 minute intervals for a period of 1½ hours. With neither dose was a secretory response observed. With the higher dose one dog showed signs of nausea. Apparently the compound is inactive at these doses since the test would have detected even weak activity. Side effects preclude testing at higher doses, but the relative activity compared with the natural tetrapeptide amide must be very low.
Therefore, charge donor properties of the side-chain of the *N*-terminal amino acid residue in this type of compounds are not involved in binding to its receptor.

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REFERENCES

1. Desaulles, P.A. and Rittel, W. (1968) in Investigations of Hypothalamic Pituitary-Adrenal Function, Memoires of the Soc. Endocrinol. (James, V.H.T. and Landon, J., eds.) no. 17, pp 125-137, Univ. Press, Cambridge.
2. Lipscomb, H.S. and Nelson, D.H. (1962) *Endocrinology* 71, 13-23.
3. Cooper, C.E. and Nelson, D.H. (1962) *J. Clin. Invest.* 41, 1599-1604.
4. Guillemin, R., Clayton, G.W., Smith, J.D. and Lipscomb, H.S. (1958) *Endocrinology* 63, 349-358.
5. Lebovitz, H.E., Genuth, S. and Pooler, K. (1966) *Endocrinology* 79, 635-642.
6. Saffran, M. and Schally, A.V. (1955) *Endocrinology* 56, 523-532.
7. Schuler, W., Schär, B. and Desaulles, P.A. (1963) *Schweiz. Med. Wschr.* 93, 1027-1030.
8. Silber, R.H., Busch, R.D. and Oslapas, R. (1958) *Clin. Chem.* 4, 278-285.
9. Guillemin, R., Clayton, G.W., Lipscomb, H.S. and Smith, J.D. (1959) *J. Lab. & Clin. Med.* 53, 830-832.
10. Jungas, R.L. and Ball, E.G. (1963) *Biochemistry* 2, 383-388.
11. Rodbell, M. (1964) *J. Biol. Chem.* 239, 375-380.
12. Dole, V.P. (1956) *J. Clin. Invest.* 35, 150-154.
13. Wieland, O. (1957) *Biochem. Z.* 329, 313-319.
14. Korn, E.D. (1955) *J. Biol. Chem.* 215, 1-26.
15. Duncombe, W.G. (1963) *Biochem. J.* 88, 7-10; *Clin. Chim. Acta* (1964) 9, 122-125.
16. Shizume, K., Lerner, A.B. and Fitzpatrick, T.B. (1954) *Endocrinology* 54, 553-560.
    Wright, R.M. and Lerner, A.B. (1960) *Endocrinology* 66, 599-609.

17. Horowitz, S.B. (1958) *J. Cell. and Comp. Physiol.* 51, 341-357.
18. Burgers, A.C.J. (1961) *Endocrinology* 68, 698-703.
19. Kastin, A.J. and Ross, G.T. (1964) *Experientia* 20, 461-462.
20. Ramachandran, J. and Lee, V. (1970) *Biochem. Biophys. Res. Commun.* 41, 358-366.
21. Hofmann, K., Andreatta, R., Bohn, H. and Moroder, L. (1970) *J. Med. Chem.* 13, 339-345.
22. Kappeler, H., Riniker, B., Rittel, W., Desaulles, P.A., Maier, R., Schär, B. and Staehelin, M. (1967) in Peptides 1966, Proceedings of the 8th European Peptide Symposium (Beyerman, H.C., Van de Linde, A. and Maassen van den Brink, W., eds.) pp. 214-220, North-Holland Publishing Co., Amsterdam.
23. Hofmann, K. manuscript in preparation.
24. Schally, A.V., Arimura, A., Bowers, C.Y., Kastin, A.J., Sawano, S. and Redding, T.W. (1968) in Recent Progress in Hormone Research (Astwood, E.B., ed.) vol. 24, pp. 497-588, Academic Press, New York.
25. Schally, A.V., Baba, Y., Arimura, A., Redding, T.W. and White, W.F. (1971) *Biochem. Biophys. Res. Commun.* 42, 50-56.
26. Schally, A.V., Arimura, A., Baba, Y., Nair, R.M.G., Matsuo, H., Redding, T.W. and Debeljuk, L. (1971) *Biochem. Biophys. Res. Commun.* 43, 393-399.
27. Matsuo, H., Baba, Y., Nair, R.M.G., Arimura, A. and Schally, A.V. (1971) *Biochem. Biophys. Res. Commun.* 43, 1334-1339.
28. Baba, Y., Matsuo, H. and Schally, A.V. (1971) *Biochem. Biophys. Res. Commun.* 44, 459-463.
29. Baba, Y., Arimura, A. and Schally, A.V. (1971) *Biochem. Biophys. Res. Commun.* 45, 483-487.
30. Yanaihara, N., Hashimoto, T., Yanaihara, C., Tsuji, K., Kenmochi, Y., Ashizawa, F., Kaneko, T., Oka, H.,

Saito, S., Arimura, A. and Schally, A.V. (1973) *Biochem. Biophys. Res. Commun.* 52, 64-73.

31. Coy, D.H., Coy, E.J. and Schally, A.V. (1973) *J. Med. Chem.* 16, 1140-1143.
32. Coy, D.H., Coy, E.J., Hirotsu, Y., Vilchez-Martinez, J.A., Schally, A.V., Van Nispen, J.W. and Tesser, G.I., (1974) *Biochemistry* 13, 3550-3553.
33. Ramirez, V.D. and McCann, S.M. (1963) *Endocrinology* 73, 193-198.
    Schally, A.V., Nair, R.M.G., Redding, T.W. and Arimura, A. (1971) *J. Biol. Chem.* 246, 7230-7236.
34. Arimura, A., Debeljuk, L. and Schally, A.V. (1972) *Endocrinology* 91, 529-532.
35. Gregory, R.A. and Tracy, H.J. (1964) *Gut* 5, 103.
36. Gregory, H., Hardy, P.M., Jones, D.S., Kenner, G.W. and Sheppard, R.C. (1964) *Nature* 204, 931-933.
37. Beacham, J., Bentley, P.H., Kenner, G.W., Mendive, J.J. and Sheppard, R.C. (1967) in Peptides 1966, Proceedings of the 8th European Peptide Symposium (Beyerman, H.C., Van de Linde, A. and Maassen van den Brink, W., eds.) pp. 235-241, North-Holland Publishing Co., Amsterdam.
38. Anderson, J.C., Barton, M.A., Gregory, R.A., Hardy, P.M., Kenner, G.W., MacLeod, J.K., Preston, J., Sheppard, R.C. and Morley, J.S. (1964) *Nature* 204, 933-934.
    Anderson, J.C., Kenner, G.W., MacLeod, J.K. and Sheppard, R.C. (1966) *Tetrahedron*, Suppl. 8, Part I, 39-51.
39. Beacham, J., Bentley, P.H., Gregory, R.A., Kenner, G.W., MacLeod, J.K. and Sheppard, R.C. (1966) *Nature* 209, 585-586.
    Morley, J.S. (1967) *J. Chem. Soc.* (C) 2410-2421.
40. Tracy, H.J. and Gregory, R.A. (1964) *Nature* 204, 935-938.

41. Morley, J.S., Tracy, H.J. and Gregory, R.A. (1965) *Nature* 207, 1356-1359.

42. Gregory, H. and Morley, J.S. (1968) *J. Chem. Soc.* (C) 910-915.
Morley, J.S. (1968) *Fed. Proc.* 27, 1314-1317.
Morley, J.S. (1967) in Peptides 1966, Proceedings of the 8th European Peptide Symposium (Beyerman, H.C., Van de Linde, A. and Maassen van den Brink, W., eds.) pp. 226-234, North-Holland Publishing Co., Amsterdam.

43. Pless, J. and Boissonnas, R.A. (1963) *Helv. Chim. Acta* 46, 1609-1625.

44. Davey, J.M., Laird, A.H. and Morley, J.S. (1966) *J. Chem. Soc.* (C) 555-566.

CHAPTER VIII

SYNTHESIS OF THE ACTH-(7-10)-TETRAPEPTIDE WITH L-ARGININE^{8} REPLACED BY L-NORARGININE

VIII. 1 INTRODUCTION

For the synthesis of [Nar^{8}]-ACTH peptides the same strategy was followed as was previously employed in the preparation of [Pmp^{9}]- and [Phe^{9}]-β-corticotrophin-(1-24)-tetracosapeptides: the suitably modified (7-10)-fragment was synthesized by stepwise or fragment condensations and subjected to the usual sequence of coupling reactions leading to the desired end-products.

Two practical ways can be envisaged for synthesizing a peptide containing norarginine: direct introduction of previously prepared norarginine or its nitro derivative, or introduction of L-α,γ-diaminobutyric acid with a temporarily masked γ-amino group which can subsequently be uncovered and then converted into a guanidino function (cf. scheme VIII, 1 and 2).

By analogy with the preparation of nitroarginine and nitrohomoarginine by treatment of the copper complexes of ornithine and lysine, respectively, with O-methyl nitroisourea in alkaline solution[1], L-α,γ-diaminobutyric acid[2] was used as the starting compound for the synthesis of the lower homologue, nitronorarginine. An improved method

for the synthesis of the rather expensive L-α,γ-diaminobutyric acid from L-glutamic acid was worked out.

For the synthesis of all intermediates required for the desired [Nar^{8}]-β-corticotrophin-(7-10)-tetracosapeptide, only standard reactions were used.

VIII. 2 SYNTHESIS AND SOME CHEMICAL PROPERTIES OF L-α,γ-DIAMINOBUTYRIC ACID

VIII. 2.1 Synthesis

The first description of the synthesis of diaminobutyric acid was given by Fischer[3]. He sequentially introduced a phthalimidoethyl residue and a bromine atom at the α-carbon of diethyl malonate, converted the product into γ-phthalimido-α-bromobutyric acid and substituted an amino group for the bromine. Hydrolysis with concentrated hydrochloric acid led to the isolation of α,γ-diaminobutyric acid *via* the crystalline oxalate.

Kurihara and Ro started with ethyl acetamidocyanoacetate and β-bromoethylphthalimide and obtained the oxalate salt of α,γ-diaminobutyric acid *via* ethyl γ-phthalimido-α-acetamido-α-cyanoacetate by acid hydrolysis[4].

Several authors have employed the Hofmann degradation of glutamine derivatives[5-8] or the dehydration and subsequent hydrogenation of asparagine derivatives[9], in order to obtain diaminobutyric acid. Other reported methods for the synthesis of

α,γ-diaminobutyric acid include:

- the reaction between ethyl acrylate and diazomethane, followed by hydrogenolysis of the isolated ethyl-2-pyrazoline-3-carboxylate and subsequent acid hydrolysis of the hydrogenated product[10].
- the Strecker reaction[11] with β-phthalimidopropaldehyde which is prepared from acrylamide and phthalimide in the presence of Triton B[12].
- the reaction between γ-butyrolactone and potassium phthalimide and the subsequent introduction of an α-bromo atom[13], or directly from α-bromo-γ-butyrolactone and potassium phthalimide[14].

The most simple methods, however, seemed to be the reaction between L-glutamic acid and hydrazoic acid (Schmidt reaction) as described by Adamson[15] and the Curtius reaction with the azides of L-glutamic acid derivatives[16,17].

Although the performance of the Schmidt reaction is experimentally simple, yields exceeding 62% have never been reported[15,18,19], mainly because of the laborious work up of the reaction mixture involved.

We improved the procedure used by Adamson in the preparation of L-α,γ-diaminobutyric acid at two points: the reaction could be brought closer to completion by very efficient mixing of the reactants, and in the work up of the reaction mixture the huge amount of sulphuric acid was neutralized with an ion exchanger instead of barium hydroxide, thus avoiding the time-consuming and troublesome filtration of the precipitated barium

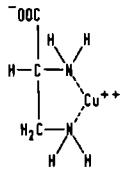
sulphate. The yield of pure L-α,γ-diaminobutyric acid, isolated as its monohydrochloride, was increased to more than 90% by these refinements.

VIII. 2.2 Chemical properties

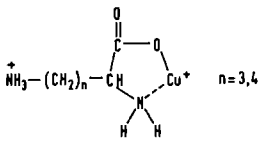
Derivatives of diaminobutyric acid, selectively acylated at N^{γ}, may be obtained from the free amino acid by direct acylation[20,21]. They are, however, more conveniently obtained by acylation of the copper complex of the amino acid[21,22], analogously to the preparation of the corresponding ornithine and lysine derivatives. Removal of copper with hydrogen sulphide yields 76-90% of the desired amino acid derivatives[21,22]; a smoother method for elimination of the copper makes use of EDTA[23,24].

The yields of the above-mentioned acylated copper complexes were in the range of 60-70% which is markedly lower than in the case of the corresponding ornithine[25] - and lysine complexes[25,26]. One explanation may be that part of the α,γ-diaminobutyric acid binds copper in the same way as α, β-diaminopropionic acid which forms mainly a type I complex whereas ornithine and lysine form only type II complexes[27].

This explanation is not supported by the high yield (92.2%) of the N^{γ}-phthaloyl-L-α,γ-diaminobutyric acid-copper complex obtained by Hase *et al.* Conversion into the free amino acid derivative, however, yielded only 58.9%[28].



type I



type II

L-nitrohomoarginine and L-nitroarginine have been prepared by treatment of L-lysine or L-ornithine with $CuCO_3$ at pH 11, followed by stirring with O-methyl nitroisourea at $0^{o}C$. A first attempt to synthesize the as yet undescribed L-nitronorarginine *via* a similar procedure was not successful. At pH 8.5, however, we could isolate the copper complex in 56% yield.

Introduction of a *t*-butyloxycarbonyl group into N^{γ}-benzyloxycarbonyl-L-α,γ-diaminobutyric acid could be achieved with Schnabel's method[29]. When the same method was applied to L-nitronorarginine, however, the thin-layer chromatograms of the reaction mixture showed six spots indicating the instability of the compound in alkaline medium. From the literature[30] it is known that the stability of L-nitroarginine is also limited at high pH values. In 2N sodium hydroxide solution at room temperature only about 2% of the decomposition product 2-nitrimino-4-carboxy-1,3-diazacycloheptane is formed, but on treatment with a 250 fold excess of sodium carbonate on a steam bath for 1½ hour, 34% of the cyclic product can be isolated. The more pronounced sensitivity of L-nitronorarginine to-

wards bases was shown in the following experiments: when a sample of the compound was treated with 1N sodium hydroxide solution at room temperature, the resulting solution was ninhydrin negative within one minute and did not reveal the characteristic UV absorption peak for the nitro group at 268 nm. On dissolution in 5% sodium carbonate the solution also became ninhydrin negative, but still showed the quenching for nitro group. The same observation was made with a solution in 5% sodium hydrogen carbonate solution. Obviously, deprotonation of the α-amino group of the zwitter-ionic form of L-nitronorarginine triggers a decomposition reaction, and since evolution of ammonia was observed, ring-closure to 2-nitrimino-4-carboxy-1,3-diazacylohexane may well be envisaged.

In disagreement with the literature[22,31], protection of the carboxylic group in N^{γ}-benzyloxycarbonyl-L-α,γ-diaminobutyric acid *via* the normal procedure with thionyl chloride and methanol[32] did not immediately result in a chromatographically homogeneous ester hydrochloride. In all experiments, varied in duration, temperature and degree of excess of thionyl chloride, a small amount of free diaminobutyric acid methyl ester dihydrochloride was found as a side-product which could be removed by chromatography on silica. The physical constants of pure methyl N^{γ}-benzyloxycarbonyl-L-α,γ-diaminobutyrate monohydrochloride were the same as found by Vogler[22].

Esterification of L-nitronorarginine could be carried out without complications.

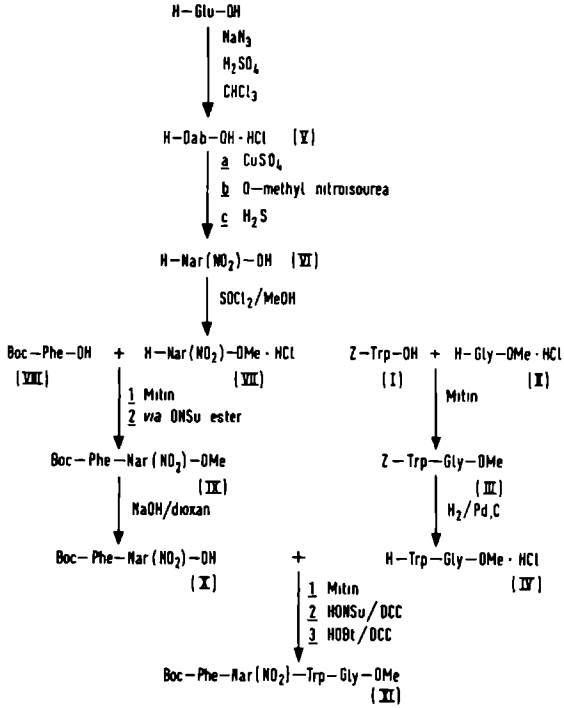
VIII. 3 METHOD OF SYNTHESIS

Boc-Phe-Nar(NO_2)-Trp-Gly-OMe (scheme VIII, 1)

Carbobenzoxylation of L-tryptophan followed by coupling with glycine methyl ester hydrochloride (II) (see chapter III) using triphenyl phosphite and imidazole[33] gave the protected dipeptide III in good yield. Catalytic hydrogenolysis (Pd/C) of this product in methanol containing one equivalent of hydrochloric acid gave the dipeptide hydrochloride IV without discolouration when pyridine was added prior to filtration of the catalyst.

L-α,γ-diaminobutyric acid (V) (see section VIII. 2.1) was converted into L-nitronorarginine (VI) in a pH-Stat at pH 8.5 with O-methyl nitroisourea. This reagent was prepared by nitration of O-methylisourea hydrogen sulphate at 0^oC[1].

L-nitronorarginine methyl ester hydrochloride (VII) was coupled with *t*-butyloxycarbonyl-L-phenylalanine (VIII) according to the method of Mitin [33] to yield the protected dipeptide IX. The dipeptide ester was also obtained by coupling VII with the 1-succinimidyl ester of VIII; both preparations had the same physical constants and gave the correct analysis. Ester hydrolysis of IX with 1.1 equivalents of base appeared not to be quantitative, but with two equivalents complete conversion was obtained.



Scheme VIII, 1

For the final condensation of the products X and IV three methods were investigated (1-3):

1) Condensation of the fragments with triphenyl phosphite as the condensing agent[33] gave no satisfactory results. There was an indication that, as well as some unidentified components, the lactam of X had been formed. Isolation of that product and dissolution in methanol gave a product with the same UV spectrum and elemental analysis as was found with the dipeptide methyl ester IX.

This result is different from that obtained by the Mitin coupling of the corresponding nitro-homoarginine peptide with IV[37]. On the other hand, when the corresponding nitroarginine peptide was coupled with methyl phenylalanyl-glycinate using triphenyl phosphite and imidazole, the physical constants of the resulting tetrapeptide differed from those found when König's method[36] was used.
At the 12th European Peptide Symposium (1972) Mitin stated[38] that because of side-reactions his method cannot be used for couplings with ω-nitroarginine derivatives as had been reported in an earlier paper[33]. The occurrence of side-reactions seems to depend on the length of the side-chain in the basic amino acid.

2) *Via* a Wünsch-Weygand coupling[34,35], about 80% of a crude product was obtained. Purification achieved by counter-current distribution gave 61% of a pure compound which gave the correct analysis.

3) Application of the method of König and Geiger[36] gave immediately a pure product on recrystallization from methanol-ether (70%).

The physical constants such as melting point, specific rotation and nitronorarginine content[39] of the main products obtained with methods 2 and 3 were different (cf. experimental section). A by-

product in condensation 2 (about 15%), however, behaved identically with the main product from method 3, which is, based on the UV data, the desired tetrapeptide.

An odd phenomenon was observed on chromatography of the main product obtained with method 2. The pure material appeared as a single spot with Rf = 0.59 (system I) on thin-layer plates if a fresh methanolic solution was employed. On chromatography of a solution which had been left for 15 minutes at room temperature, a second spot appeared on the chromatogram with Rf = 0.40. After a longer period of standing only the latter spot was found. On evaporation of a solution from which the original compound had disappeared, a residue was left which had again Rf = 0.59 on chromatography in system I. It underwent the same transformation on standing in methanol as described above. Apparently the process is completely reversible and repeatable. The compound with Rf = 0.59 gave Ehrlich's reaction, whereas the product with Rf = 0.40 could not be detected with this reagent. Both spots gave strong UV fluorescence quenching and chlorine/tolidine reactions.

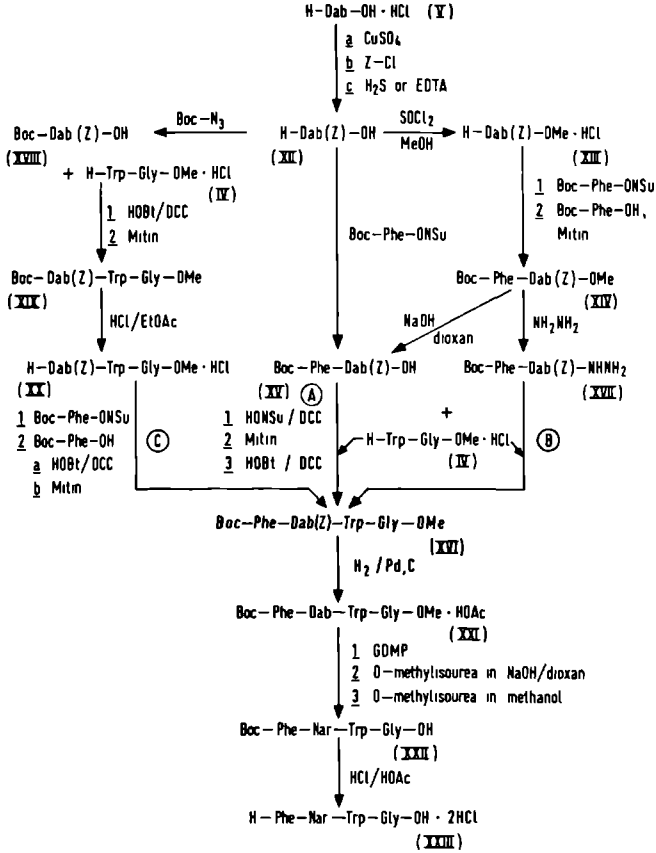
The product obtained with method 3 and the by-product in condensation 2 had a Rf value of 0.30 (system I) and did not change on standing.

H-Phe-Nar-Trp-Gly-OH.2HCl (scheme VIII, 2)

N^{γ}-benzyloxycarbonyl-L-α,γ-diaminobutyric acid

was obtained *via* its copper complex by treatment with hydrogen sulphide or the disodium salt of ethylenediamine tetraacetic acid[23,24]. The method of Kuwata[23] was, in our hands, only successful when an excess of EDTA was used instead of an equimolar amount. A slight modification of Eckstein's procedure[24] appeared to give good results. The product XII was acylated directly with the *t*-butyloxycarbonyl-L-phenylalanine-1-succinimidyl ester in the presence of an organic base and 1-hydroxybenzotriazole. The optical purity of the protected dipeptide XV was demonstrated by comparison with samples obtained by a similar acylation of the methyl ester XIII (see section VIII. 2.2), using König's as well as Mitin's method, and subsequent alkaline hydrolysis of the protected dipeptide ester XIV.

The fragment condensation of the partially protected dipeptides XV and IV (route Ⓐ) with *N*-hydroxysuccinimide/*N*,*N*'-dicyclohexylcarbodiimide as the condensing reagent gave a product having physical constants differing from those of the product obtained by the same fragment condensation *via* Mitin's method (see table VIII. 1). Therefore, attempts were made to synthesize the tetrapeptide derivative *via* another pathway, to find out which of the products from the fragment condensation might be desired tetrapeptide XVI. An azide coupling (Ⓑ) between the crystalline hydrazide XVII, obtained in good yield from the methyl ester XIV and hydrazine provided, however,



Scheme VIII, 2.

no solution to the problem (see table VIII. 1).

Two other possible synthetic procedures, *viz.* a fragment condensation using 1-hydroxybenzotriazole and dicyclohexylcarbodiimide, and a stepwise procedure were then investigated. In the latter experiment N^{γ}-benzyloxycarbonyl-L-α,γ-diaminobutyric acid (XII) was converted into the N^{α}-*tert*-butyloxycarbonyl derivative according to the meth-

Table VIII-1

Physical and analytical data of the protected tetrapeptide Boc-Phe-Dab(Z)-Trp-Gly-OMe prepared *via* different routes

	Fragment condensation				Stepwise procedure		
	HONSu DCC	Mitin	azide	HOBt DCC	*via* ONSu	HOBt DCC	Mitin
Yield (%)	57	75	53	75	75	59	76
M.p. (^{o}C)	186-188	171-173.5	183.5-188	181-182	179-181	182.5-184.5	183.5-185.5
$[\alpha]_D$(c = 1.0, MeOH)	-33.5^{o}	-28.0^{o}	-29.5^{o}	-33.2^{o}*	-33.2^{o}*	-33.3^{o}	-32.7^{o}
(c = 1.0, DMF)	-14.0^{o}	-19.6^{o}	-18.4^{o}	-18.8^{o}	-18.5^{o}	-19.8^{o}	-18.6^{o}
Analysis:							
$C_{40}H_{48}N_6O_9$ (756.86)							
% C (63.48)	63.4	63.3	63.2	63.1	63.2	63.2	62.9
% H (6.39)	6.4	6.6	6.4	6.6	6.6	6.4	6.4
% N (11.10)	10.9	10.8	10.8	11.1	10.9	11.0	11.1

* These rotations were measured with a Perkin-Elmer 241 apparatus, the others on a Zeiss polarimeter.

od of Schnabel[29] and the product XVIII was isolated as a dicyclohexyl ammonium salt. The optical rotation, in methanol as well as in dimethylformamide, was quite different from that reported by Vogler[40] and Arold[41], but it had the same melting point as given by Vogler. Coupling of XVIII with IV, gave identical products when performed according to König or *via* Mitin's procedure. So, it seemed permissible to suppose that the protected tripeptide XIX had been formed in both cases. The *N*-terminal protective group was removed with hydrogen chloride in ethyl acetate and the resulting product (XX) was then coupled with *t*-butyloxycarbonyl-L-phenylalanine (route Ⓒ). This coupling step was performed with the 1-succinimidyl ester of VIII and also *via* König's and Mitin's method. Again, identical products were isolated from all three procedures (see table VIII. 1).
The results of this stepwise procedure were in good agreement with those obtained *via* the fragment condensation method using 1-hydroxybenzotriazole and dicyclohexylcarbodiimide; therefore we feel confident that these products are the pure, desired protected tetrapeptide ester XVI.

Hydrogenation of the protected tetrapeptide XVI in the presence of palladium on charcoal as the catalyst was carried out in acetic acid as the solvent.

The introduction of the amidino group into the side-chain of XXI can be carried out using several reagents. Bodanszky used 1-guanyl-3,5-dimethyl-

pyrazole[42] in the synthesis of N^{α}-benzyloxycarbonyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycine from the corresponding ornithine peptide[43]. For this purpose, 2.31 mmoles of the reagent were added to a dimethylformamide solution containing one mmole of peptide and about 4.3 mmoles of triethylamine. After 4 hours at about 40°C and 60 hours at room temperature, addition of water precipitated 88.8% of the desired pentapeptide. Following exactly this procedure, we did not succeed, however, in isolating the norarginine-containing tetrapeptide ester in reasonable purity and yield.

Amidination with a large excess (∿150 equivalents) of O-methylisourea at high pH (10-12) in sodium hydroxide solution as applied to large peptides[44] and proteins[45], also failed to give *t*-butyloxycarbonylphenylalanyl-norarginyl-tryptophyl-glycine in reasonable yield and purity.

Better results were obtained when a freshly prepared methanolic solution of O-methylisourea[46] was added to a solution of the tetrapeptide XXI in methanol. In 1934 Kapfhammer and Müller[47] synthesized several α-guanidino amino acids and dipeptides in this way, and Greenstein[48] prepared homoarginyl-glutamic acid from lysyl-glutamic acid using the same method.

When O-methylisourea was liberated from its salt[46] and the amidination reaction carried out in methanol, it appeared that the norarginine peptide arose as the free carboxylic acid since alkaline hydrolysis of the product after removal of the *t*-butyloxycarbonyl group did not modify the peptide.

When XXI was treated with 5 equivalents of O-methylisourea in methanol for 2-3 days at room temperature, and water was added to the mixture, some 60% of crude XXII could be isolated. About 75% was obtained when 20 equivalents of the free urea derivative were used and the reaction mixture was held at a temperature of 0-4^{o}C for some 20 hours. Purification of the norarginine tetrapeptide could be achieved by chromatography on Kieselgel 60 using isopropyl alcohol-ammonia-water (8:1:1) as the eluent.

Removal of the N^{α}-protective *t*-butyloxycarbonyl group was carried out with hydrochloric acid in acetic acid; precipitation with ether gave the free tetrapeptide XXIII in good yield.

VIII. 4 EXPERIMENTAL SECTION

For details concerning the abbreviations, thin-layer chromatography and the performance of measurements, see the appendices.

Scheme VIII, 1.

Z-Trp-OH (I)

The synthesis of this compound was carried out using Z-Cl and NaOH at 0°C. Recrystallization was achieved with ethyl acetate/petroleum ether.

Yield : 95% Lit.[49] 85-98%
M.p. : 124-125°C 126°C
$[\alpha]_D^{24}$: +24.6° (c = 1.0, EtOAc) (Zeiss) -
- 8.9° (c = 1.0, MeOH)
+ 2.9° (c = 1.0, HOAc)
TS : Rf = 0.07 (A), = 0.68 (B), = 0.56 (E) (UV and R-H)
Analysis:
$C_{19}H_{18}N_2O_4$ (338.36) Calcd.: % C 67.45 % H 5.36 % N 8.28
Found : % C 67.3 % H 5.3 % N 8.3

Z-Trp-Gly-OMe (III)

Coupling of I and II (see chapter III) was carried out with imidazole and triphenyl phosphite[33]. The product was recrystallized from methanol.

		Lit.[33]	[50]	[51]
Yield:	92%	97%	74%	59%
M.p. :	157.5-158.5°C	157-158°C	156-158°C	158-159°C
$[\alpha]_D^{24}$:	-13.7° (c = 1.0, HOAc) (Zeiss)	-12.7° (c = 2, HOAc)	-11.0° (c = 3, HOAc)	-11.0° (c = 2, HOAc)

TS : Rf = 0.46 (A), = 0.72 (B), = 0.77 (E) (UV and R-H)

Analysis:

$C_{22}H_{23}N_3O_5$	Calcd.:	% C 64.54	% H 5.66	% N 10.26
(409.44)	Found :	% C 64.5	% H 5.7	% N 10.3

H-Trp-Gly-OMe.HCl (IV)

Compound III was suspended in methanol and hydrogenated in the presence of palladium on charcoal and one equivalent of aqueous concentrated HCl. During the uptake of hydrogen the ester went into solution. Then one equivalent of pyridine was added and the catalyst was removed by filtration. The filtrate was evaporated, the residue taken up in methanol and evaporated again; after repeating this procedure, the off-white non-crystalline salt was isolated and kept in the dark.

Yield	: 98%	Lit.[52] 91%
M.p.	: 99-101°C (dec.)	-
$[\alpha]_D^{27}$	: +48.8° (c = 1.0, 1N HCl) (Zeiss) +29.2° (c = 1.0, MeOH)	+48.6° (c = 1.1, 1N HCl) for the hemihydrate
TS	: Rf = 0.09 (A), = 0.37 (B) (UV, ninh. and R-H)	

Analysis:

$C_{14}H_{18}N_3O_3Cl$	Calcd.:	% C 53.94	% H 5.82	% N 13.48
(311.77)	Found :	% C 54.4	% H 5.9	% N 13.6

H-Dab-OH.HCl (V)

100 mmoles of L-glutamic acid (14.72 g) and 70 ml of H_2SO_4 (about 100%) were brought into a conical reaction flask (370 ml) with a wide, ground joint (B 55) which was equipped with a three way adaptor holding a reflux condensor, the stirring rod of a very efficient stirring apparatus (Vibro-Mischer, model E 1 from "A.G. für Chemie-Apparatenbau", Switzerland; plunger diameter of 45 mm, 17 mm from the walls of the vessel) and a stopper. After the amino acid had been dissolved by stirring and gentle heating, 70 ml of chloroform were added, followed by one equi-

valent of NaN_3 (6.50 g) in one portion. The temperature increased within a few seconds to the boiling point of chloroform and was maintained between 50° and 62°C during the course of the reaction. Another equivalent of NaN_3 was added after one, four and six hours. After eight hours the reaction mixture was cooled to room temperature and poured into enough distilled water to obtain a half molar H_2SO_4 solution. After separating from the $CHCl_3$ layer, the aqueous solution was filtered slowly through a column of Dowex 50X2 (700 ml of wet Dowex with a capacity of 0.8 meq/ml resin were used). The filtrate appeared to be completely free of diaminobutyric acid and was discarded. The column was washed with water until the pH of the effluent was about 5 and the product was then eluted with 4 M ammonia. The filtrate was evaporated, the residue dissolved in water, treated with charcoal and evaporated again. The residue was then dissolved in a small amount of HCl, the pH adjusted to 5 with pyridine and the product precipitated with 96% ethanol.

Yield	: 92%	Lit.[9] -	[53] -	[54] -
M.p.	: 226-227°C	225°	224-226°C	225°
$[\alpha]_D^{28}$	: +22.5° (c = 1.0, 6N HCl) (Zeiss)	+25.3° (c = 1.1, 5N HCl)	+22.0°	+23.8° (c = 1.2, 6N HCl)

TS : Rf = 0.30 (L), = 0.25 (W) (ninh. and R-H)

Analysis:

$C_4H_{11}N_2O_2Cl$	Calcd.:	% C 31.08	% H 7.17	% N 18.12
(154.60)	Found :	% C 31.0	% H 7.0	% N 18.2

H-Nar(NO_2)-OH (VI)

7.73 g (50 mmoles) of V were dissolved in 60 ml of water. 4.00 g (25 mmoles) of $CuSO_4$ were added to the solution with stirring and the reaction mixture cooled. The pH of the solution was adjusted to 8.5 and kept constant during the reaction with the aid of an autotitrator loaded

with 4N NaOH. Subsequently 6.66 g (56 mmoles) of O-methyl nitroisourea (prepared according to the method of Heyboer[1] in 30% yield with m.p. 118.5-119.5°C) were added in the course of a few hours. After about 4 hours, the pH was adjusted to 7 with acetic acid and the light-blue copper complex was filtered, washed with water and acetone and dried with ether (57%).

The complex was dissolved in dilute hydrochloric acid and the amino acid was liberated by saturation of the solution with H_2S. After filtration of the precipitated CuS and concentration of the filtrate, the pH was brought to 6 and VI was isolated as a white crystalline solid which was purified by recrystallization from water. Yield: 62%. M.p.: 206.5-208.5°C (dec. 160-175°C). TS: Rf = 0.88 (L), = 0.27 (U) (UV and R-H). $[\alpha]_D^{23}$ = +35.6° (c = 1.0, 1N HCl), = +35.2° (c = 1.0, 2N HCl) (Zeiss). ε = 15,810 (at λ_{max} = 268.0 nm in DMF/0.2 N HCl, 1:1 v/v), ε = 1,810 (at λ_{max} = 265.0 nm in TFA)[39].

Analysis:

$C_5H_{11}N_5O_4.H_2O$	Calcd.:	% C 26.91	% H 5.87	% N 31.38
(205.17)	Found :	% C 26.8	% H 5.8	% N 31.3

H-Nar(NO_2)-OMe.HCl (VII)

Esterification of VI with $SOCl_2$ and MeOH was carried out in the usual way. The white compound was isolated in about 99% yield. M.p.: 91-93°C. TS: Rf = 0.10 (B), = 0.60 (U) (UV, ninh. and R-H). $[\alpha]_D^{26}$ = +18.1° (c = 1.0, MeOH) (Zeiss). A = 0.41 at λ_{max} = 268.5 nm (concentration: 0.008 mg/ml in DMF-0.2N HCl, 1:1 v/v) and A = 0.70 at λ_{max} = 260.0 (concentration 0.1 mg/ml in TFA). Elemental analysis indicated the presence of a variable amount (up to 1.5 equivalents) of hydrochloric acid.

Boc-Phe-Nar(NO_2)-OMe (IX)

1) Boc-Phe-OH (VIII, see chapter III) (5 mmoles = 1.327

g), VII (5 mmoles = 1.278 g), *N*-ethylmorpholine (5 mmoles + the amount necessary for neutralization of the excess of HCl present in VII), imidazole (7.5 mmoles = 0.511 g) and triphenyl phosphite (7.5 mmoles = 2.327 g) were dissolved in about 15 ml of DMF and kept at 40°C for 18 hours. Then the solvent was evaporated and the residue was taken up in EtOAc. The solution was extracted with 5% $NaHCO_3$ solution, water, 5% citric acid solution, water and finally with saturated NaCl solution. The organic layer was dried on Na_2SO_4, filtered and evaporated. Crystallization of the remaining oil from ether gave IX in 71% yield. M.p.: 122-124°C. TS: Rf = 0.21 (A), = 0.70 (B), = 0.45 (I) (UV and R-H). $[\alpha]_D^{25}$ = -4.8° (c = 1.0, EtOAc), = -9.5° (c = 1.0, MeOH) (Zeiss). ε = 15,920 (at λ_{max} = 269.0 nm in DMF/0.2N HCl, 1:1 v/v), ε = 1,540 (at λ_{max} = 261.5 nm in TFA). Nitronorarginine content: 45% (theoretically 43.9%)[39].

Analysis:

$C_{20}H_{30}N_6O_7$	Calcd.: % C 51.49	% H 6.48	% N 18.02
(466.49)	Found : % C 51.3	% H 6.5	% N 18.0

2) Coupling of Boc-Phe-ONSu (see chapter III) with VII gave, after purification, the protected dipeptide ester IX with the same physical constants, in 69% yield.

Boc-Phe-Nar(NO_2)-OH (X)

The addition of 1.1 equivalents of NaOH to a solution of IX in dioxan did not lead to complete hydrolysis. At least 2 equivalents were necessary for complete saponification which was then accomplished within half an hour. Evaporation of the solvents and dissolution of the residue in EtOAc was followed by extraction with 5% citric acid. The organic layer was washed with water and saturated NaCl

solution, and dried on Na_2SO_4. After filtration, evaporation of the solvent and drying, 92% of X was obtained. M.p.: 109-113°C (dec.). TS: Rf = 0.50 (B) (UV and R-H). $[\alpha]_D^{25}$ = -3.2° (c = 1.0, MeOH) (Zeiss). ε = 13,920 (at λ_{max} = 268.5 nm in DMF/0.2N HCl, 1:1 v/v), ε = 1,790 (at λ_{max} = 260.5 nm in TFA). Nitronorarginine content: 40% (theoretically 45.1%).

Analysis:

$C_{19}H_{28}N_6O_7$	Calcd.: % C 50.44	% H 6.24	% N 18.57
(452.47)	Found : % C 50.1	% H 6.2	% N 18.2

Boc-Phe-Nar(NO_2)-Trp-Gly-OMe (XI)

HOBt/DCC method

The procedure finally adopted ran as follows: to a solution of 4.28 mmoles (1.937 g) of X in 15 ml of DMF were added with stirring 4.28 mmoles (1.335 g) of IV, 0.54 ml (one equivalent) of *N*-ethylmorpholine and 6.50 mmoles (0.878 g) of HOBt. The reaction mixture was cooled in an ice bath and 0.972 g (4.71 mmoles) of DCC were added. After stirring at 0°C for 2 hours and at room temperature for another 2½ hours, the precipitated DCU was filtered and the solvent evaporated. The residue was taken up in EtOAc and was washed and dried as described before (see IX) 89% of the not quite homogeneous product was obtained. Recrystallization from methanol/ether gave about 70% of pure XI. M.p.: 120-122°C. TS: Rf = 0.74 (B), = 0.30 (I) (UV, R-H and Ehrlich). $[\alpha]_D^{28}$ = -20.5° (c = 1.0, MeOH) (Zeiss). ε = 22,310 (at λ_{max} = 270.0 nm in DMF/0.2N HCl, 1:1 v/v), ε = 7,370 (at λ_{max} = 263.5 nm in TFA). Nitronorarginine content: 30% (theoretically 28.9%).

Analysis:

$C_{33}H_{43}N_9O_9.H_2O$	Calcd.: % C 54.46	% H 6.23	% N 17.32
(727.78)	Found : % C 54.6	% H 6.2	% N 17.4

After drying a sample in a high vacuum over P_2O_5 at 80°C the following values were found:

$C_{33}H_{43}N_9O_9$	Calcd.:	% C 55.84	% H 6.11	% N 17.76
(709.76)	Found :	% C 55.5	% H 6.0	% N 17.6

The first experiments were carried out with HONSu. One equivalent of DCC was added, at -20°C, to the reaction mixture consisting of equimolar amounts of IV, X and Et_3N, and 2 equivalents of HONSu. The temperature was allowed to rise spontaneously to room temperature. Working up of the reaction mixture as described in the previous preparation gave about 80% of a chromatographically inhomogeneous product, which could not be purified by recrystallization from ethyl acetate/petroleum ether. Counter-current distribution with the system methanol-ethyl acetate-petroleum ether-water (5:4:6:3) gave, however, a pure product (K = 0.13, r_{max} = 31 after 275 transfers). Yield: 2.247 g (starting from 2.299 g of X). M.p.: 94-97°C. TS: Rf = 0.59 (I) (UV, R-H and Ehrlich) for a fresh solution (see previous section). $[\alpha]_D^{21}$ = -15.6° (c = 1.0, MeOH) at t = o and -14.0° after 8 hours (Zeiss). A = 0.68 at λ_{max} = 274.0 nm (concentration 2.04×10^{-2} mg/ml DMF-0.2N HCl, 1:1 v/v) and A = 0.82 at λ_{max} = 262.0 nm (concentration 0.098 mg/ml TFA). Nitronorarginine content, calculated for XI: 37% (theoretical value 28.9%).

Analysis for:

$C_{33}H_{43}N_9O_9 \cdot H_2O$	Calcd.:	% C 54.46	% H 6.23	% N 17.32
(727.78)	Found :	% C 54.1	% H 6.2	% N 17.4

Some 50 mg of another pure product were obtained from the counter-current distribution (K = 0.05, r_{max} = 14) possessing the same properties as the product obtained *via* the HOBt/DCC method. M.p.: 119-122°C. TS: Rf = 0.30 (I) (UV, R-H and Ehrlich). ε = 22,250 (at λ_{max} = 270.0 nm in DMF-0.2N HCl, 1:1 v/v), ε = 7,475 (at λ_{max} = 266.0 nm in TFA). Nitronorarginine content: 29% (theoretically 28.9%).

Analysis:

$C_{33}H_{43}N_9O_9 \cdot \frac{1}{2}H_2O$	Calcd.:	% C 55.14	% H 6.17	% N 17.54
(718.77)	Found :	% C 55.2	% H 6.1	% N 17.1

Scheme VIII, 2.

H-Dab(Z)-OH (XII)

50 mmoles (7.74 g) of H-Dab-OH.HCl (V) and 25 mmoles of $CuSO_4$ (4.00 g) were dissolved in about 40 ml of H_2O. The solution was cooled and the pH adjusted to 10.5. Subsequently 60 mmoles of Z-Cl (8.53 ml) were slowly added with stirring; the pH of the solution was kept constant with a pH Stat loaded with 4N NaOH. After completion of the addition, stirring and cooling were continued for 5 hours. Then, the blue copper complex of XII was filtered and washed with water, acetone and ether. Yield: 67%.

Two methods were used for the removal of the copper:

a) H_2S was bubbled through a solution of the copper complex in dilute hydrochloric acid for about 3 hours. The precipitated CuS was filtered off with hyflo and the pH of the filtrate was then adjusted to about 7 with diethylamine. After cooling and filtering, the compound was recrystallized from EtOH-H_2O (1:1).

Yield	: 70%	Lit.[22]	76%	[32] -	[55] 18%
M.p.	: 226-227.5°C		238-240°C	238°C	235°C
$[\alpha]_D^{25}$	: +19.9° (c = 1.0, 1N HCl) (Zeiss)		-	+12.5°	-

TS : Rf = 0.40 (B) (ninh. and R-H)

Analysis:

$C_{12}H_{16}N_2O_4$	Calcd.:	% C 57.13	% H 6.39	% N 11.10
(252.27)	Found :	% C 57.2	% H 6.3	% N 11.3

b) 7.00 g of the copper complex were suspended in water. 13.80 g of EDTA (3 equivalents) were added to the sus-

pension followed by HCl to a pH of 2.5. Addition of a large amount of water with stirring resulted in a clear solution which was neutralized by the addition of solid NaOH. Concentration of the solution to about 10% of its original volume gave a precipitate of XII which was recrystallized from EtOH-H_2O (1:1), containing a small amount of EDTA. Yield: 71%, m.p. 216-218°C, $[\alpha]_D^{21}$ = +21.0° (c = 0.4, 2N HCl) (Perkin-Elmer 241). The product gave the expected elemental analysis.

H-Dab(Z)-OMe.HCl (XIII)

When the esterification was carried out according to the method of Vogler and Lanz[22], a small amount of H-Dab(H)-OMe.2HCl was obtained as a by-product. Changes in the excess of $SOCl_2$ used, the temperature or the duration of the reaction did not prevent the formation of the by-product. Removal of the impurity was achieved by filtration of the reaction mixture through silica gel (Kieselgel 60) with $CHCl_3$-MeOH (9:1) as the eluent. The pure product precipitated on addition of ether to a concentrated solution of XIII in methanol.

Yield	: 73%	Lit.[22] 80%	[32]-	[56] 90%
M.p.	: 157-158°C	164-166°C	140°C	166-168°C
$[\alpha]_D^{22}$	: +15.5°	+15.2°	+20.8°	+15.4°
	(c = 1.0, MeOH)	(c = 2,	(c = 1,	(c = 0.48,
	(Zeiss)	MeOH)	MeOH)	MeOH)

TS : Rf = 0.40 (B), = 0.33 (I), = 0.55 (V) (ninh. and R-H)

Analysis:

$C_{13}H_{18}N_2O_4$.HCl	Calcd.:	% C 51.57	% H 6.33	% N 9.25
(302.76)	Found :	% C 51.4	% H 6.2	% N 9.2

Boc-Phe-Dab(Z)-OMe (XIV)

1) 6.37 mmoles (2.306 g) of Boc-Phe-ONSu were dissolved in 25 ml of DMF, and 6.37 mmoles (1.930 g) of XIII and

6.37 mmoles (0.89 ml) of Et_3N were added with stirring. After 3 hours water was added and the resulting oil was extracted into ethylacetate. The organic layer was washed with an aqueous solution of 3-dimethylaminopropyl amine, water, citric acid solution and water. After drying on Na_2SO_4, the solution was concentrated and petroleum ether was added. After cooling and filtration 81.5% of XIV was obtained. M.p.: 117.5-118.5°C. $[\alpha]_D^{27}$ = -21.7° (c = 1.0, MeOH) (Zeiss). TS: Rf = 0.60 (A), = 0.74 (B) (R-H).

Analysis:

$C_{27}H_{35}N_3O_7$	Calcd.:	% C 63.14	% H 6.87	% N 8.18
(513.59)	Found :	% C 63.0	% H 7.0	% N 8.1

2) In 12 ml of DMF were dissolved: 10 mmoles (2.653 g) of Boc-Phe-OH, 10 mmoles (3.028 g) of XIII, 10 mmoles (1.40 ml) of Et_3N, 15 mmoles (1.021 g) of imidazole and 15 mmoles (4.654 g) of triphenyl phosphite. After 20 hours at 40°C, a small amount of $Et_3N.HCl$ was filtered off and the filtrate dripped into 150 ml of water. The precipitate was filtered, washed extensively with water and ether and dried. Yield: 74.3% of a chromatographically homogeneous material. M.p.: 116.5-117.5°C. $[\alpha]_D^{27}$ = -22.6° (c = 1.02, MeOH) (Zeiss).

Analysis: Found : % C 62.8 % H 6.8 % N 8.2

Boc-Phe-Dab(Z)-OH (XV)

a) 6.04 mmoles (3.102 g) of XIV were suspended in 37 ml of a mixture of dioxan-water (2:1). 12.1 ml of a 1N NaOH solution were added with stirring. After 20 minutes, citric acid solution and EtOAc were added and the organic layer was washed with water and dried over Na_2SO_4. Addition of petroleum ether to the concentrated EtOAc solution, filtration and drying gave 90.0% of XV. M.p.: 111.5-112.5°C. $[\alpha]_D^{24}$ = -24.9° (c = 1.0, EtOAc) (Zeiss).

TS: Rf = 0.67 (B) (R-H).

Analysis:

$C_{26}H_{33}N_3O_7$	Calcd.:	% C 62.51	% H 6.66	% N 8.41
(499.56)	Found :	% C 62.5	% H 6.8	% N 8.5

b) 4.0 mmoles (1.450 g) of Boc-Phe-ONSu, 4.0 mmoles (1.009 g) of XII and 4.0 mmoles (0.505 ml) of *N*-ethylmorpholine were suspended in 30 ml of DMF. After the addition of 6.0 mmoles (0.811 g) of HOBt, the suspension was stirred for 20 hours at room temperature. Then the pH of the (now clear) solution was adjusted to 3 with 2N $KHSO_4$ solution, and EtOAc was added. The organic layer was washed with water and dried. After evaporation of the solvent the residue was crystallized from EtOAc/petroleum ether: yield 80%. Tlc revealed that a small amount of HONSu was still present. Therefore, the product was suspended in 100 ml of water and the suspension was stirred for some 16 hours. Filtration gave pure XV with m.p. 111-112.5°C. $[\alpha]_D^{21}$ = -26.3° (c = 0.6, EtOAc) (Perkin-Elmer 241).

Analysis:

$C_{26}H_{33}N_3O_7.H_2O$	Calcd.:	% C 60.40	% H 6.82	% N 8.13
(517.58)	Found :	% C 60.4	% H 6.6	% N 8.3

Boc-Phe-Dab(Z)-NHNH$_2$ (XVII)

2.568 g (5.0 mmoles) of Boc-Phe-Dab(Z)-OMe were dissolved in 30 ml of methanol. At room temperature, 1.46 ml of $N_2H_4.H_2O$ (30 mmoles) were added. After 17 hours, the white precipitate was filtered and washed with methanol. Yield: 93.7%. M.p.: 168.5-170.5°C. $[\alpha]_D^{23}$ = -21.6° (c = 1.0, DMF) (Zeiss). TS: Rf = 0.33 (A), = 0.70 (B) (R-H and Barton).

Analysis:

$C_{26}H_{35}N_5O_6$	Calcd.:	% C 60.80	% H 6.87	% N 13.64
(513.59)	Found :	% C 60.7	% H 7.0	% N 13.7

Boc-Phe-Dab(Z)-Trp-Gly-OMe (XVI)

Fragment condensation

Ⓐ 1. HONSu/DCC method[34,35]

14.81 mmoles (7.400 g) of XV, 14.81 mmoles (1.63 ml) of *N*-ethylmorpholine and 29.62 mmoles (3.409 g) of HONSu were dissolved in 60 ml of DMF. After cooling at -12°C, 3.056 g (14.81 mmoles) of DCC were added with stirring. The temperature was allowed to rise spontaneously to room temperature. After 2 hours a solution of 11.11 mmoles (3.464 g) of IV (3/4 equivalents) and 11.11 mmoles (1.22 ml) of *N*-ethylmorpholine in 10 ml of DMF were added, and stirring was continued for 15 hours at room temperature. Then, the precipitated DCU was removed by filtration and the filtrate was evaporated. Dissolution of the residue in EtOAc and extraction of the organic layer with water, 3-dimethylaminopropyl amine solution, water, 5% citric acid solution and water, followed by drying and evaporation gave XVI which was contaminated with a small amount of DCU. Treatment with methanol-ether gave pure XVI in about 57% yield. TS: Rf = 0.41 (A), = 0.76 (B) (UV, R-H and Ehrlich). For physical constants, see table VIII, 1.

2. Mitin's method[33]

The same procedure as described for the synthesis of XIV (*via* route 2) was employed in the preparation of the tetrapeptide XVI. The physical and analytical data (see table VIII, 1) did not change on treatment of the product with methanol-ether.

3. HOBt/DCC method[36]

2.20 mmoles (1.099 g) of Boc-Phe-Dab(Z)-OH were dissolved in 5 ml of DMF. To this solution were added

2.20 mmoles of IV (0.686 g), 2.20 mmoles of *N*-ethylmorpholine (0.278 ml) and 3.30 mmoles (0.446 g) of HOBt with stirring. The solution was then cooled at 0°C and 2.20 mmoles (0.453 g) of DCC were added. After 2 hours at 0°C the reaction mixture was stirred at room temperature for about 4 hours. The same work up of the reaction mixture as described in procedure 1. yielded 75% of the tetrapeptide XVI. For physical and analytical data see table VIII, 1.

(B) Azide coupling

4.00 mmoles (2.054 g) of XVII were dissolved in 25 ml of DMF and the solution was cooled to -15°C. Then, 11.0 mmoles of HCl in EtOAc were added (5.67 ml of a 1.94 N solution) followed by 4.80 mmoles of *t*-butyl nitrite (0.547 ml). After 15 minutes at -15°C, 11.0 mmoles of *N*-ethylmorpholine (1.21 ml) were added with stirring followed by 4.80 mmoles (1.500 g) of IV and 4.80 mmoles of *N*-ethylmorpholine (0.53 ml). The temperature of the reaction mixture was allowed to rise spontaneously to 0°C. The solution was then kept in the refrigerator for about 23 hours. After filtration and evaporation of the solvent the residue was taken up in EtOAc and the solution was extracted as usual. Tlc revealed that there were still some by-products present. Therefore, the extraction procedure was repeated. Drying and evaporation gave a solid which was extracted with a small amount of warm EtOAc; the solution was decanted and cooled. The insoluble product and the precipitate from the cooled solution appeared to be homogeneous on tlc. For physical and analytical data, see table VIII, 1.

Boc-Dab(Z)-OH (XVIII)

H-Dab(Z)-OH (5.00 mmoles = 1.261 g) was suspended in 20 ml of dioxan and 20 ml of water. The pH was adjusted to 10.5 and kept constant with a pH Stat loaded with NaOH solution. Boc-N_3[57] (0.82 ml; 25% excess) was added in three portions with stirring. Stirring was continued for one day. Then the solution was extracted twice with ether and acidified with citric acid solution. The amino acid derivative was extracted into ethyl acetate and the organic layer was washed with water and with saturated NaCl solution and dried. Evaporation gave 80% of a colourless oil. TS: Rf = 0.67 (B) (R-H). $[\alpha]_D^{23}$ = -14.7° (c = 2.0, MeOH), = -20.5° (c = 1.0, DMF), = -12.5° (c = 1.0, 96% HOAc).

To a solution of 4.00 mmoles of the oil (1.405 g) in 10 ml of ether 5.00 mmoles of DCHA (1.0 ml) were added. After cooling overnight the white precipitate was filtered, dried and recrystallized from ether.

Yield :	88%	Lit.[40] -	[41]93%
M.p. :	99-102°C	98-101°C	121-122°C
$[\alpha]_D^{23}$:	∿0° (c = 1.0, DMF)	-6.2°	-18.2°
	(Zeiss)	(c = 1, DMF)	(c = 2, MeOH)
	∿0° (c = 1.0, MeOH)		
	-8.3° (c = 1.0, HOAc)		

TS : Rf = 0.68 and 0.60 (dec.) (B) (R-H)

Analysis:

$C_{29}H_{47}N_3O_6$	Calcd.: % C 65.26	% H 8.88	% N 7.88
(533.71)	Found : % C 65.4	% H 9.2	% N 7.7

Boc-Dab(Z)-Trp-Gly-OMe (XIX)

1) 3.30 mmoles (1.154 g) of XVIII were dissolved in 10 ml of DMF. To this solution were added 3.30 mmoles (1.029 g) of IV, 3.30 mmoles (0.418 ml) of *N*-ethylmorpholine and 4.95 mmoles (0.669 g) of HOBt. The reaction mixture was cooled at 0°C and then 3.30 mmoles (0.681 g)

of DCC were added with stirring. After 2 hours at 0°C the mixture was stirred for 4 hours at room temperature and then cooled; the precipitated DCU was removed by filtration. After evaporation of the filtrate, the residue was dissolved in EtOAc and the solution extracted with 2N $KHSO_4$ solution, H_2O, 5% $NaHCO_3$ solution, H_2O and saturated NaCl solution. After drying on Na_2SO_4 the solvent was evaporated and the residue crystallized from methanol-ether giving the protected tripeptide XIX as a slightly yellow-coloured precipitate in 73.5% yield. M.p.: 152-153°C. $[\alpha]_D^{21}$ = -28.8° (c = 0.86, MeOH) (Zeiss). TS: Rf = 0.44 (A), = 0.76 (B) (UV, R-H and Ehrlich).

Analysis:

$C_{31}H_{39}N_5O_8$	Calcd.:	% C 61.07	% H 6.45	% N 11.49
(609.68)	Found :	% C 60.7	% H 6.6	% N 11.4

2) The same procedure as described for the synthesis of XIV (*via* route 2) was employed here. Extensive washings of the isolated product with ether, water and again with ether gave 68.3% of XIX with m.p. 154-155.5°C. $[\alpha]_D^{22}$ = -28.9° (c = 1.0, MeOH) (Zeiss). TS: Rf = 0.42 (A), = 0.75 (B) (UV, R-H and Ehrlich).

Analysis: Found : % C 61.2 % H 6.3 % N 11.2

H-Dab(Z)-Trp-Gly-OMe.HCl (XX)

Elimination of the Boc group from XIX was achieved with 20 equivalents of HCl. Progression of the deprotection was followed by tlc (system A). As soon as the starting material had disappeared, N_2 was bubbled through the solution for about 5 minutes and the solution was concentrated *in vacuo* at room temperature. After cooling, ether was added with stirring. The precipitate was filtered, washed extensively with ether and dried. Yield: 85-95%. M.p.: 142-146°C (dec. at about 180°C). $[\alpha]_D^{26}$ = +9.7° (c = 1.0, MeOH), =

$+3.6^{o}$ (c = 1.0, DMF) (Perkin-Elmer 241); = $+8.7^{o}$ (c = 1.0, MeOH) (Zeiss). TS: Rf = 0.50 (B) (UV, ninh. and R-H).

Analysis:

$C_{26}H_{32}N_5O_6Cl$	Calcd.:	% C 57.19	% H 5.91	% N 12.83
(546.02)	Found :	% C 56.7	% H 5.9	% N 12.8

Boc-Phe-Dab(Z)-Trp-Gly-OMe (XVI)

Stepwise condensation

Ⓒ 1. Using Boc-Phe-ONSu

4.77 mmoles of XX (2.605 g) were dissolved in 20 ml of DMF. (When the pH of the solution dropped below 4.5 *N*-ethylmorpholine was added to bring it back again). A further equivalent of *N*-ethylmorpholine (0.60 ml) was then added, followed by 4.77 mmoles (1.729 g) of Boc-Phe-ONSu. The reaction mixture was kept at room temperature for about 5 hours. Then the solution was cooled, water was added with stirring and the precipitated tetrapeptide XVI filtered and washed with water. Dissolution of the compound in methanol and precipitation with ether gave pure XVI (see table VIII, 1).

2a. *Via* the HOBt/DCC method

3.00 mmoles (1.638 g) of XX were dissolved in 18 ml of DMF. *N*-ethylmorpholine (0.378 ml) was added, followed by 3.00 mmoles (0.796 g) of Boc-Phe-OH and 4.50 mmoles (0.608 g) of HOBt. At $0^{o}C$ 3.30 mmoles (0.681 g) of DCC were added and the reaction mixture was stirred for 1.5 hours at $0^{o}C$ and for 2 hours at room temperature. Then the precipitated DCU was filtered after cooling and the filtrate was evaporated. Dissolution of the residue in EtOAc and extraction as usual gave nearly homogeneous XVI. Further purification was achieved by adding a small amount of

MeOH to the dried product, filtration of undissolved material, and drying. Ether was added to the filtrate and the precipitated product was filtered and dried. The combined yields amounted to 59%. The first crop was used to determine the physical and analytical data (see table VIII, 1).

2b. *Via* Mitin's method

2.60 mmoles (1.414 g) of XX, 2.60 mmoles (0.690 g) of Boc-Phe-OH, 2.60 mmoles (0.328 ml) of *N*-ethylmorpholine, 3.90 mmoles (0.266 g) of imidazole and 3.90 mmoles (1.210 g) of triphenyl phosphite were dissolved in 10 ml of DMF and kept at 40°C for about 22 hours. Then the solution was poured into water and the resulting precipitate filtered after cooling. Washing with water and ether, followed by recrystallization from methanol-ether gave XVI (see table VIII, 1).

Boc-Phe-Dab-Trp-Gly-OMe.HOAc (XXI)

Hydrogen was bubbled through a solution of XVI in 90% HOAc for about half an hour. Then the catalyst (Pd/C) was filtered off with hyflo and the solvent was evaporated. The residue was freed from acetic acid by evaporation with isopropyl alcohol. Dissolution of the residue in as little isopropyl alcohol as possible and addition of diisopropyl ether gave XXI in yields varying between 88 and 95%. TS: Rf = 0.44 (B), = 0.17 (E), = 0.55 (F) (UV, ninh., R-H and Ehrlich). In nearly every preparation traces of one or two unknown by-products were found which were almost impossible to remove. It appeared to be desirable to use the product in the following preparation without delay.

Boc-Phe-Nar-Trp-Gly-OH (XXII)

To 7.5 ml of a freshly prepared methanolic solution (about 4 mmoles/ml) of O-methylisourea obtained from the

hydrogen sulphate according to the method of Stieglitz and McKee[46] in about 70% yield, were added 961 mg of XXI (1.41 mmoles). The solution was kept at 0-4°C for about one day, then concentrated and cooled. Water was added slowly (about 7-10 times the volume of the solution) with rubbing. The precipitate was removed by centrifugation, washed once with water and dried. Yield of impure XXII: 717 mg. For purification the product was subjected to chromatography on Kieselgel 60 using isopropyl alcohol-ammonia-water (8:1:1) as the eluent. The combined fractions containing the Sakaguchi positive XXII were evaporated, the residue dissolved in methanol and the turbid solution filtered through hyflo. Addition of ethyl acetate to the concentrated methanolic solution gave the pure tetrapeptide in 40% yield. M.p.: about 185°C (dec.). $[\alpha]_D^{21}$ = -17.6° (c = 0.58, MeOH) (Perkin-Elmer 241). TS: Rf = 0.52 (F), = 0.32 (T), = 0.50 (W) (UV, R-H, Sakaguchi and Ehrlich). Amino acid analysis (6N HCl, 110°, 24 hours): Trp 0.77 (1), Nar 1.04 (1), Gly 1.09 (1), Phe 1.00 (1).

Analysis:

$C_{32}H_{42}N_8O_7.2H_2O$	Calcd.: % C 55.96	% H 6.75	% N 16.32
(686.77)	Found : % C 56.3	% H 6.8	% N 16.0

H-Phe-Nar-Trp-Gly-OH.2HCl (XXIII)

Removal of the N^{α}-protective group was performed in 1.6N HCl/HOAc (about 15-20 equivalents of HCl). After 10 minutes ether was added. The precipitate formed was filtered after cooling and extensively washed with ether. Yield: about 92%. M.p.: 187-190°C. $[\alpha]_D^{21}$ = +5.4° (c = 1.23, 90% HOAc) (Perkin-Elmer 241). TS: Rf = 0.24 (F), = 0.48 (W). (UV, ninh., R-H and Ehrlich). After drying a sample in a high vacuum over P_2O_5 at 80°C, the substance was equilibrated with air moisture. UV spectrum: A = 0.621 at λ_{max} = 280.0 nm (concentration: 3.92 mg in 50 ml 0.1N HCl). Peptide content: about 78%[58].

Analysis:

$C_{27}H_{36}N_8O_5Cl_2 \cdot 4H_2O$	Calcd.:	% C 46.62	% H 6.38
(695.60)	Found :	% C 46.5	% H 6.3
	Calcd.:	% N 16.11	% Cl 10.19
	Found :	% N 15.7	% Cl 9.9

REFERENCES

1. Heyboer, N., Heymens Visser, G. and Kerling, K.E.T. (1962) *Rec. Trav. Chim.* 81, 69-72.
2. For a survey see: Greenstein, J.P. and Winitz, M. (1961) in Chemistry of the amino acids, vol. 3, pp. 2469-2475, J. Wiley, New York.
3. Fischer, E. (1901) *Ber. Deutschen Chem. Ges.* 34, 2900-2906.
4. Kurihara, T. and Ro, K. (1956) *Ann. Rept. Tohoku Coll. Pharm.* 3, 77-81; *C.A.* (1957) 51, 5698d.
5. Karrer, P., Escher, K. and Widmer, R. (1926) *Helv. Chim. Acta* 9, 301-323.
6. Synge, R.L.M. (1939) *Biochem. J.* 33, 671-678.
7. King, F.E. and Kidd, D.A.A. (1949) *J. Chem. Soc.* 3315-3319.
8. Bruckner, V., Kovacs, J. and Kovacs, K. (1953) *J. Chem. Soc.* 1512-1514.
9. Zaoral, M. and Rudinger, J. (1959) *Coll. Czech. Chem. Commun.* 24, 1993-2012.
10. Carter, H.E., Van Abeele, F.R. and Rothrock, J.W. (1949) *J. Biol. Chem.* 178, 325-334.
11. Strecker, A. (1850) *Justus Liebigs Ann. Chem.* 75, 27.
12. Atkinson, R.O. and Poppelsdorf, F. (1952) *J. Chem. Soc.* 2448.
13. Zaoral, M. (1959) *Coll. Czech. Chem. Commun.* 24, 1314-1319.

14. Talbot, G., Gaudry, R. and Berlinguet, L. (1958) *Can. J. Chem.* 36, 593-596.
15. Adamson, J.W. (1939) *J. Chem. Soc.* 1564-1568.
16. Akabori, S. and Numano, S. (1932) *J. Chem. Soc. Japan* 53, 213-217; *Bull. Chem. Soc. Japan* (1936) 11, 214-217.
17. LeQuesne, W.J. and Young, G.T. (1950) *J. Chem. Soc.* 1954-1959.
18. Martha Pigretti, M. and Stoppani, A.O.M. (1961) *Anales Asoc. Quim. Arg.* 49, 55-74; 161-176.
19. Mushakwar, I.K. and Koeppe, R.E. (1963) *J. Biol. Chem.* 238, 2460-2463.
20. Zaoral, M., Rudinger, J. and Šorm, F. (1953) *Coll. Czech. Chem. Commun.* 18, 530-549. *Chem. Listy* (1953) 47, 427-439.
21. Poduska, K. and Rudinger, J. (1959) *Coll. Czech. Chem. Commun.* 24, 3449-3467.
22. Vogler, K. and Lanz, P. (1960) *Helv. Chim. Acta* 43, 270-279.
23. Kuwata, S. and Watanabe, H. (1965) *Bull. Chem. Soc. Japan* 38, 676-677.
24. Eckstein, H., Sievers, R.E. and Bayer, E. (1973) *Justus Liebigs Ann. Chem.* 1467-1470.
25. Ledger, R. and Stewart, F.H.C. (1965) *Aust. J. Chem.* 18, 933-935.
26. Tesser, G.I. (1961) Thesis, Groningen.
27. Albert, A. (1952) *Biochem. J.* 50, 690-697.
28. Hase, S., Schwartz, I.L. and Walter, R. (1972) *J. Med. Chem.* 15, 126-128.
29. Schnabel, E. (1967) *Justus Liebigs Ann. Chem.* 702, 188-196.
30. Paul, R., Anderson, G.W. and Callahan, F.M. (1961) *J. Org. Chem.* 26, 3347-3350.
31. Kurihara, T. and Suzuki, K. (1955) *J. Pharm. Soc. Japan* 75, 1269-1272.

32. Brenner, M. and Huber, W. (1953) *Helv. Chim. Acta* 36, 1109-1115.
33. Mitin, Y.V. and Glinskaya, O.V. (1969) *Tetrahedron Letters* 5267-5270.
34. Wünsch, E. and Drees, F. (1966) *Chem. Ber.* 99, 110-120.
35. Weygand, F., Hoffmann, D. and Wünsch, E. (1966) *Z. Naturforschg.* 21b, 426-428.
36. König, W. and Geiger, R. (1971) in Peptides 1969, Proceedings of the 10th European Peptide Symposium (Scoffone, E., ed.) pp. 17-22, North-Holland Publishing Co., Amsterdam;
Chem. Ber. (1970) 103, 788-798.
37. Tesser, G.I. Pleumekers, A.W.J., Bassie, W. and Balvert-Geers, I.C. (1973) *Rec. Trav. Chim.* 92, 1210-1222.
38. Mitin, Y.V., Gudkov, A.T., Zapevalova, N.P. and Maximov, E.E. (1973) in Peptides 1972, Proceedings of the 12th European Peptide Symposium (Hanson, H. and Jakubke, H.-D., eds.) pp. 57-64, North-Holland Publishing Co., Amsterdam.
39. Gamper, J.R. (1962) *Helv. Chim. Acta* 45, 1418-1425.
40. Vogler, K., Studer, R.O., Lanz, P., Lergier, W. and Böhni, E. (1965) *Helv. Chim. Acta* 48, 1161-1177.
41. Arold, H. (1969) *J. für Prakt. Chem.* 311, 278-285.
42. Habeeb, A.F.S.A. (1960) *Can. J. Biochem. and Physiol.* 38, 493-501.
43. Bodanszky, M., Ondetti, M.A., Birkhimer, C.A. and Thomas, P.L. (1964) *J. Amer. Chem. Soc.* 86, 4452-4459.
44. Borin, G., Toniolo, C., Moroder, L., Marchiori, F., Rocchi, R. and Scoffone, E. (1972) *Int. J. Peptide Protein Res.* 4, 37-45.
45. Chervenka, C.H. and Wilcox, P.E. (1956) *J. Biol. Chem.* 222, 635-647.
46. Stieglitz, J. and McKee, R.H. (1900) *Ber. Deutschen Chem. Ges.* 33, 1517-1519.

47. Kapfhammer, J. and Müller, H. (1934) *Z. Physiol. Chem.* 225, 1-12.
48. Greenstein, J.P. (1935) *J. Biol. Chem.* 109, 541-544.
49. Smith, E.L. (1948) *J. Biol. Chem.* 175, 39-47.
50. Geiger, R., Sturm, K. and Siedel, W. (1963) *Chem. Ber.* 96, 1080-1087.
51. Hofmann, K., Woolner, M.E., Spühler, G. and Schwartz, E.T. (1958) *J. Amer. Chem. Soc.* 80, 1486-1489.
52. Hofmann, K. and Lande, S. (1961) *J. Amer. Chem. Soc.* 83, 2286-2289.
53. Kawasaki, H. (1960) *Nippon Kagaku Zasshi* 81, 280-282; *C.A.* (1961) 55, 6393i.
54. Wilkinson, S. (1951) *J. Chem. Soc.* 104-108.
55. Takagi, S., Tsukatani, H. and Hayashi, K. (1959) *Chem. Pharm. Bull.* (Tokyo) 7, 619-621.
56. Poduska, K. (1966) *Coll. Czech. Chem. Commun.* 31, 2955-2972.
57. Carpino, L.A., Giza, C.A. and Carpino, B.A. (1959) *J. Amer. Chem. Soc.* 81, 955-957.
58. Beaven, G.H. and Holiday, E.R. (1952) in Advances in Protein Chemistry (Anson, M.L., Bailey, K. and Edsall, J.T., eds.) vol. VII, pp. 319-386, Academic Press, New York.

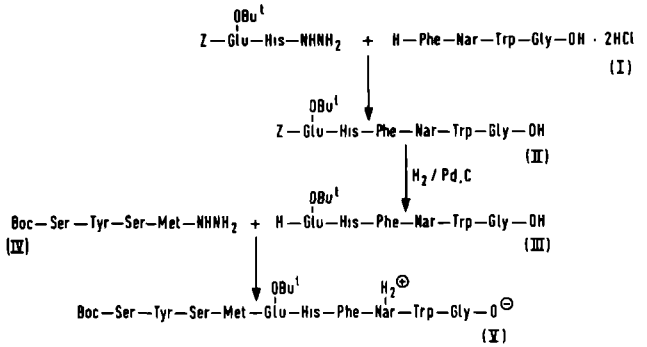
CHAPTER IX

SYNTHESIS AND BIOLOGICAL ACTIVITY OF TWO ACTH ANALOGUES CONTAINING L-NORARGININE IN POSITION 8

IX. 1 INTRODUCTION AND METHOD OF SYNTHESIS

Two possible reaction pathways leading to the corticotrophin-(5-10)-hexapeptide, H-Glu(OBu^t)-His-Phe-Nar-Trp-Gly-OH, can be envisaged. One of them starts from the fully protected tetrapeptide Boc-Phe-Nar(NO_2)-Trp-Gly-OMe, described in chapter VIII, from which the α-amino protective *t*-butyloxycarbonyl group is cleaved with hydrogen chloride. Acylation with the dipeptide Z-Glu(OBu^t)-His-N_3 does indeed give the pure protected hexapeptide ester in reasonable yield, but subsequent saponification of the methyl ester with sodium hydroxide in aqueous dioxan results in a product which stubbornly resists complete catalytic hydrogenation. It seems that only purification by counter-current distribution prior to the hydrogenation step guarantees a slow but complete conversion of such a hexapeptide[1].

The alternative pathway starts from the corresponding free tetrapeptide which is likewise acylated with Z-Glu(OBu^t)-His-N_3 (scheme IX, 1). The partially protected hexapeptide II underwent hydrogenolytic cleavage of the N^{α}-protective function without difficulties. The resulting product (III)

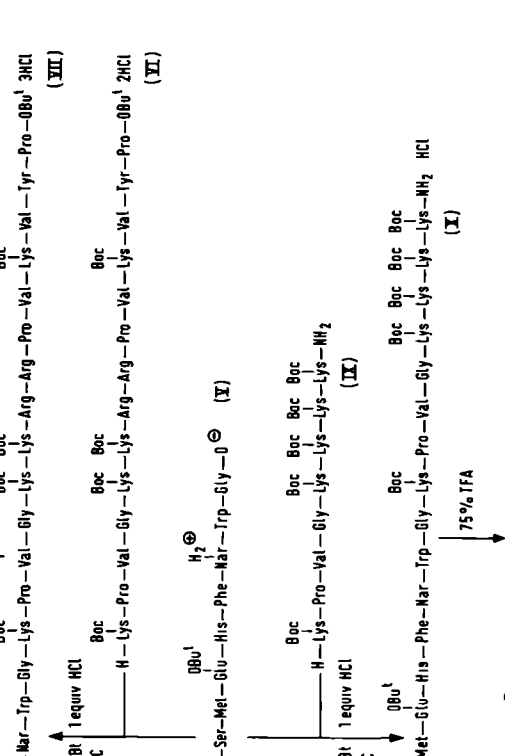


Scheme IX, 1

was used in the now familiar reaction sequence (cf. chapters III and IV) leading to the desired end-products: the azide coupling with the tetrapeptide IV was done according to the method of Honzl and Rudinger[2] (scheme IX, 1).
The partially protected, zwitter-ionic decapeptide was then extended to a tetracosapeptide as well as an octadecapeptide amide by coupling with the relevant protected peptides VI and IX, respectively (see scheme IX, 2). In both cases König's method[3] was used. In the latter case the amino acid sequence of the resulting octadecapeptide amide differed not only at position 8 but also at the positions 17 and 18 from the natural sequence in ACTH. It is known, however, that substitution of Arg^{17} and Arg^{18} by Lys does not lower the biological potency of ACTH analogues[4].

The products obtained from these couplings were purified by counter-current distribution,

then deprotected with 75% trifluoroacetic acid, and the resulting trifluoroacetates converted into acetates in the usual way.



Scheme IX, 2

IX. 2 EXPERIMENTAL SECTION

For details concerning the abbreviations, thin-layer chromatography and the performance of measurements, see the appendices.

Z-Glu(OBu^t)-His-Phe-Nar-Trp-Gly-OH (II)

A sample of I was completely freed from hydrochloric acid by addition of sufficient *N*-ethylmorpholine and then supplied with an excess (1.5 equivalents were used) of Z-Glu(OBu^t)-His-$NHNH_2$. The coupling was performed as described in chapter III. After standing at 0°C for about 40 hours, the reaction mixture was concentrated and cooled, and water was added with stirring. The precipitate was filtered and recrystallized from acetic acid/ether giving 58% of a chromatographically homogeneous product. TS: Rf = 0.41 (F), = 0.64 (L), = 0.53 (W) (UV, R-H, Pauly and Ehrlich). M.p.: 208-210°C (dec.).
$[\alpha]_D^{21}$ = -17.0° (c = 1.0, 90% HOAc).
Analysis:

$C_{50}H_{62}N_{12}O_{11}.HOAc.H_2O$	Calcd.:	% C	57.55	% H	6.32	% N	15.49
(1085.19)	Found :	% C	56.5	% H	6.1	% N	15.5

H-Glu(OBu^t)-His-Phe-Nar-Trp-Gly-OH (III)

Hydrogenation of II was carried out in 90% HOAc with palladium on charcoal as the catalyst. After 3 hours the catalyst was removed by filtration and fresh Pd/C was added; hydrogenation was continued for another 3 hours. Then the reaction mixture was filtered, the filtrate was concentrated and cooled, and ether was added dropwise with stirring. The isolated product (85% yield) had an Rf of 0.10 (F) and 0.59 (L) (UV, R-H and Ehrlich). It was contaminated with a trace of a slower-running by-product of unknown constitution.

Boc-Ser-Tyr-Ser-Met-Glu(OBu^t)-His-Phe-Nar-Trp-Gly-OH (V)

The azide coupling between the acetate of III and an excess of IV (1.5 equivalents) was carried out as described before. After 20 hours at 0°C the reaction mixture was concentrated and acetonitrile was added with stirring. After cooling, the precipitate was filtered, washed with acetonitrile and dried giving 90% of V. For further purification, the compound was dissolved in DMF and precipitated with acetonitrile with stirring. Yield: 78.3%. M.p.: 183-187°C. TS: Rf = 0.26 (B), = 0.10 (C), = 0.37 (F), = 0.60 (P) (UV, R-H, Pauly, Sakaguchi, Barton and Ehrlich). $[\alpha]_D^{21}$ = -16.1° (c = 1.05, DMF), = -19.0° (c = 0.7, 90% HOAc).

Boc-Ser-Tyr-Ser-Met-Glu(OBu^t)-His-Phe-Nar-Trp-Gly-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-OBu^t (VII)

244 mg of V were condensed with 443 mg (1.2 equivalents) of VI with the aid of 2 equivalents of HOBt and 1.5 equivalents of DCC as described in chapter III. After 21 hours the reaction mixture was concentrated and cooled, and the precipitated DCU filtered. Peroxide-free ether was added to the filtrate with stirring. After cooling, the resulting precipitate was filtered giving 608 mg of crude VII. The product was purified by counter-current distribution with the system methanol-chloroform-carbon tetrachloride-buffer (8:5:2:4) (buffer composition: 19.25 g of NH_4OAc and 28.5 ml of HOAc made up to 1000 ml with H_2O). After 243 transfers 260 mg (about 45%) of pure VII were obtained (K = 0.47, r_{max} = 78). TS: Rf = 0.29 (B), = 0.16 (C), = 0.43 (F), = 0.74 (P) (R-H, Pauly, Sakaguchi, Ehrlich and Barton). M.p.: 175-180°C (dec.). $[\alpha]_D^{21}$ = -52.7° (c = 0.60, MeOH). UV spectrum in 90% HOAc (concentration 0.18 mg/ml): A = 0.385 at λ_{max} = 277.6 nm.

[Nar^{8}]-β-corticotrophin-(1-24)-tetracosapeptide

Deprotection of VII was carried out with 75% TFA under N_2 in the dark for 3 hours. The solution was then cooled at about -30°C and peroxide-free ether was added dropwise with stirring. After 10 minutes the precipitate was filtered, washed with ether and dried. Conversion of the trifluoroacetate into the acetate was carried out with a weakly basic ion exchanger (Merck II - acetate form). The filtrate was lyophilized.

TA: Rf = 0.60 (P) (R-H). TC: Rf = 0.49 (P), = 0.60 (R) (ninh. and R-H).

$[\alpha]_D^{21}$ = -85.0° (c = 0.60, 1% HOAc), $[\alpha]_{578}$ = -88.7°, $[\alpha]_{546}$ = -101.2°, $[\alpha]_{436}$ = -176.2° and $[\alpha]_{365}$ = -295.0°.

Amino acid analysis after complete hydrolysis (6N HCl, 24 hours at 110°C) (theoretical values in parentheses): Trp 0.64 (1), Lys 3.67 (4), His 0.99 (1), NH_3 0.55, Nar + Arg 3.03 (3), Ser 1.56 (2), Glu 1.00 (1), Pro 2.94 (3), Gly 2.00 (2), Val 3.20 (3), Met 1.18 (1), Tyr 2.02 (2), Phe 1.01 (1).

UV spectra: in 0.1 N HCl: A = 0.661 at λ_{max} = 275.9 nm (concentration 0.307 mg/ml),
in 0.1 NaOH : A = 0.716 at λ_{max} = 288.5 nm and A = 0.691 at λ = 282.0 nm (concentration 0.245 mg/ml),
Tyr/Trp ratio: 2.0[5]. Peptide content: 72.5 ± 0.08% (mean of 2 values) as determined spectrophotometrically according to Beaven and Holliday[6].

H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-NH_2 (IX)

Compound IX was obtained from the benzyloxycarbonyl derivative according to the method of Riniker and Rittel[7]. The product IX was precipitated by the addition of water

to a concentrated methanolic solution of IX.

Yield: 83%	Lit.[7]	88%
M.p. : about 195°C (dec.)		∿220°C (dec.)
$[\alpha]_D^{21}$: -33.6° (c = 1.0, MeOH)		-33° (c = 1, MeOH)
TS : Rf = 0.60 (F), = 0.69 (I) (ninh. and R-H).		

Boc-Ser-Tyr-Ser-Met-Glu(OBu^t)-His-Phe-Nar-Trp-Gly-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-NH$_2$ (X)

The octadecapeptide amide X was synthesized according to the method of Riniker and Rittel[7] using HOBt instead of HONSu. The crude material was subjected to counter-current distribution using the system methanol-chloroform-carbon tetrachloride-buffer (10:7:4:3) (buffer composition: 7.7 g of NH_4OAc and 57.1 ml of HOAc made up to 1000 ml with H_2O). After 160 transfers 135 mg of a pure product (about 48%) were obtained (K = 0.86, r_{max} = 74). TS: Rf = 0.48 (C), = 0.61 (F), = 0.76 (P) (R-H, Pauly, Sakaguchi, Ehrlich and Barton).
M.p.: 195-200°C (dec.). $[\alpha]_D^{21}$ = -29.7° (c = 0.43, 90% HOAc). UV spectrum in 90% HOAc: A = 0.393 at λ_{max} = 278.2 nm (concentration 0.217 mg/ml).

[Nar^8, Lys^{17,18}]-β-corticotrophin-(1-18)-octadecapeptide amide (XI)

The same deprotection procedure as described for the preparation of the tetracosapeptide VIII was employed. Tlc and electrophoresis of the resulting product revealed the presence of a by-product which could be completely separated, however, from XI by preparative high voltage paper electrophoresis*.

* We should like to thank Dr. W. de Jong (Biochemistry Department, Nijmegen) for performing the electrophoresis experiment.

Thin-layer chromatography of the acid hydrolysate of the isolated by-product revealed that it was unreacted octapeptide amide IX.

TA: Rf = 0.25 (P); TC: Rf = 0.44 (P) (ninh. and R-H).

$[\alpha]_D^{21} = -56.4^o$ (c = 0.50, 1% HOAc), $[\alpha]_{578} = -58.8^o$, $[\alpha]_{546} = -67.0^o$ and $[\alpha]_{436} = -116.4^o$.

Amino acid analysis after complete hydrolysis (6N HCl, 24 hours at 110°C) (theoretical values in parentheses): Lys 4.40 (5), His 1.06 (1), NH_3 1.55 (1), Nar 0.97 (1), Ser 1.46 (2), Glu 0.95 (1), Pro 1.03 (1), Gly 2.00 (2), Val 1.02 (1), Met 0.97 (1), Tyr 0.90 (1), Phe 0.94 (1).

UV spectra: in 0.1 N HCl: A = 0.627 at λ_{max} = 278.8 nm (concentration 0.291 mg/ml).
in 0.1 N NaOH: A = 0.526 at λ_{max} = 281.5 nm and A = 0.516 at λ = 288.2 nm (concentration 0.227 mg/ml). Tyr/Trp ratio: 0.9[5].

IX. 3 BIOLOGICAL ACTIVITY

The results of preliminary *in vivo* experiments (see chapter VII) with [Nar^8]-ACTH-(1-24) are given in table IX-1.

Table IX-1

Plasma corticosterone (γ/100 ml blood) in 24 hours hypophysectomized rats, corrected for blanc value (4 rats/group).

	Dose	Time following injection			
	(mg/kg)	15 min.	30 min.	60 min.	90 min.
ACTH-(1-24)	1.0	19 ± 2	34 ± 4	33 ± 5	16 ± 3
[Nar^8]-ACTH-(1-24)	1.0	17 ± 2	15 ± 2	13 ± 3	1.6± 2

Although *in vivo* experiments are less reliable than *in vitro* experiments, one can conclude that the Nar^{8}-peptide has a significant ACTH activity.

The melanocyte-stimulating activity of the two Nar^{8}-peptides has been investigated with lizard skin (see chapter VII). [Nar^{8}]-ACTH-(1-24) had an activity of 0.003-0.01 (α-MSH was set at 1) and [Nar^{8}, $Lys^{17,18}$]-ACTH-(1-18)-NH_2 of 0.006-0.01. Compared with the normal tetracosapeptide, both compounds possess about 20-30% of the MSH activity of ACTH-(1-24).

REFERENCES

1. Tesser, G.I., Pleumekers, A.W.J., Bassie, W. and Balvert-Geers, I.C. (1973) *Rec. Trav. Chim.* 92, 1210-1222.
2. Honzl, J. and Rudinger, J. (1961) *Coll. Czech. Chem. Commun.* 26, 2333-2344.
3. König, W. and Geiger, R. (1970) *Chem. Ber.* 103, 788-798.
4. Desaulles, P.A., Riniker, B. and Rittel, W. (1969) in Protein and Polypeptide Hormones, Proceedings of the International Symposium, Liège, 1968 (Margoulies, M., ed.) pp. 489-491, Excerpta Medica Foundation, Amsterdam.
5. Bencze, W.L. and Schmid, K. (1957) *Anal. Chem.* 29, 1193-1196.
6. Beaven, G.H. and Holiday, E.R. (1952) in Adv. in Protein Chemistry (Anson, M.L., Bailey, K. and Edsall, J.T., eds.) vol. VII, pp. 319-386, Academic Press, New York.
7. Riniker, B. and Rittel, W. (1970) *Helv. Chim. Acta* 53, 513-519.

Appendix A

ABBREVIATIONS

The abbreviations used for the amino acid residues and the notation of peptides are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature, Biochem. J. (1972) 126, 773-780.

List of other abbreviations:

ACTH	adrenocorticotrophic hormone
Boc	*t*-butyloxycarbonyl
1-BuOH	1-butanol
Dab	L-α,γ-diaminobutyric acid
DCC	*N,N'*-dicyclohexylcarbodiimide
DCHA	dicyclohexylamine
DCU	*N,N'*-dicyclohexylurea
DMF	*N,N*-dimethylformamide
DMSO-d_6	deuterated dimethylsulphoxide
Et_3N	triethylamine
EtOAc	ethyl acetate
EtOH	ethyl alcohol
GOMP	1-guanyl-3,5-dimethylpyrazole
Har	L-homoarginine
HOBt	1-hydroxybenzotriazole
HONp	*p*-nitrophenol
HONSu	*N*-hydroxysuccinimide
m	meta
MeOH	methyl alcohol
Msc	methylsulphonylethyloxycarbonyl
α-MSH	α-melanocyte-stimulating hormone
Nar	L-norarginine (L-α-amino-γ-guanidinobutyric acid)
o	ortho
OBu^t	*t*-butyloxy

OMe	methoxy
OTcp	2,4,5-trichlorophenoxy
p	para
Pmp	L-pentamethylphenylalanine
i-ProOH	isopropyl alcohol
sec.	secondary
t	tertiary
TA	thin-layer chromatography on alumina
TC	thin-layer chromatography on cellulose
TFA	trifluoroacetic acid
Tlc	thin-layer chromatography
TMS	trimethylsilane
TS	thin-layer chromatography on silica
Z	benzyloxycarbonyl

Appendix B

TLC DATA

For thin-layer chromatographical analyses, pre-coated silica gel (TS), alumina (TA) and cellulose (TC) plates with a fluorescence indicator of Merck (F. 254) were used, unless otherwise stated.

To develop the chromatograms, the following solvent systems were used:

A = benzene-acetone (1:1)
B = 1-butanol-acetic acid-water (10:1:3)
C = ethyl acetate-pyridine-acetic acid-water (62:21:6:11)
D = acetic acid-0.1 N hydrochloric acid (4:1)
E = chloroform-methanol-acetic acid (95:20:3)
F = 1-butanol-acetic acid-water (4:1:1)
G = chloroform-methanol (4:1)
H = 2-propanol-formic acid-water (20:1:5)
I = chloroform-methanol (9:1)
J = *sec.*-butyl alcohol-acetic acid-water (67:10:23)
K = *sec.*-butyl alcohol-isopropyl alcohol-9% monochloroacetic acid (58:8:34)
L = chloroform-methanol-17% ammonia (5:5:1)
M = 1-butanol-pyridine-acetic acid-water (4:1:1:2)
N = 1-butanol-acetic acid-water (1:2:1)
O = 1-butanol-pyridine-acetic acid-water (16:3:1:4)
P = 1-butanol-pyridine-acetic acid-water (38:24:8:30)
Q = 1-butanol-pyridine-acetic acid-water (42:24:4:30)
R = 1-butanol-pyridine-formic acid-water (40:24:6:30)
S = chloroform-methanol-acetic acid (40:10:0.15)
T = isopropyl alcohol-25% ammonia-water (8:1:1)
U = phenol-water (3:1)
V = chloroform-methanol (1:1)
W = 1-propanol-25% ammonia (3:2)

The pyridine present on plates developed in the pyridine containing systems was removed by spraying the dried plates with concentrated ammonia solution and subsequent heating at about 100°C.

Methods used for the detection of components

UV	quenching of fluorescence after exposure of the plates at 254 nm.
ninh.	ninhydrin reagent for detection of free amino groups.
R-H	Reindel-Hoppe test (chlorine/*o*-tolidine reagent) for -NH- groups.
Pauly	colouration of histidine containing compounds.
Ehrlich	specific reagent for tryptophan containing compounds.
Sakaguchi	detection of free guanidino groups.
Barton	colouration of hydrazides and tyrosine.

Reagents and experimental techniques

Ninh. : 240 mg of ninhydrin in 400 ml of 1-butanol and 16 ml of acetic acid. After spraying, the plates were heated in an oven at 80-100°C for 5-10 minutes.

R-H : silica gel plates were exposed to chlorine for about 15 seconds and the excess of chlorine was removed by spraying with ethanol; alumina and cellulose plates were sprayed with acetic acid followed by a dilute sodium hypochlorite solution. The spots could be visualized by spraying with a reagent composed of a solution of 4 g of *o*-tolidine in 80 ml of acetic acid and 320 ml of water, mixed with a solution of 3.36 g of potassium iodide in 400 ml of water.

Pauly : a freshly prepared solution of diazotized sulphanilic acid (about 50 mg of the moist compound) in 10 ml of 5% Na_2CO_3 was used.

Ehrlich : the plates were sprayed with a solution of 1 g of 4-dimethylaminobenzaldehyde in 25 ml of concentrated HCl and 75 ml of methanol and then heated.

Sakaguchi: the chromatograms were sprayed with a solution of 0.02 g of α-naphtol, 2.5 g of NaOH and 4 g of urea in 50 ml of water and dried. Then, a solution of 0.33 ml of bromine and 2.5 g of NaOH in 50 ml of water was used as the second spray reagent.

Barton : equal volumes of a solution of 30 g of $FeCl_3$ in 170 ml of water and of a solution of 2 g of $K_3Fe(CN)_6$ in 198 ml of water were mixed just before use.

Appendix C

MEASUREMENTS

Melting points given in chapters II and VIII were determined with a Heiztisch Microscope (Leitz), those in other chapters with a Totolli apparatus (Büchi). They are uncorrected.

Specific rotations were measured with a Perkin-Elmer 141 polarimeter unless otherwise stated. In chapter IX only a Perkin-Elmer 241 apparatus was used.

The UV spectra mentioned in chapters II, III and VIII were recorded with a Beckman DK-2A apparatus. The tetracosapeptides in chapter III were measured with a Perkin-Elmer 402 spectrophotometer. A Cary 15 apparatus was used for recording the spectra mentioned in chapters IV and IX. In all measurements, closed quartz cells of 1 cm pathlength were used.

Amino acid analyses were carried out with a Technicon TSM 1 apparatus at the laboratories of Ciba-Geigy Ltd., Basle, Switzerland.

N.M.R. spectra were recorded on a Varian HA-100 apparatus with TMS as internal reference.

SAMENVATTING

Omvangrijke onderzoekingen betreffende struktuur-funktie relaties van het adrenocorticotrope hormoon hebben aan het licht gebracht, dat van dit 39 aminozuren omvattende peptide-hormoon niet minder dan 21 aminozuren vanaf het carboxyl uiteinde verwijderd kunnen worden zonder verlies van de biologische werkzaamheid. Het resterende oktadekapeptide vertoont volledige ACTH-aktiviteit.

Daarnaast is vastgesteld dat op diverse plaatsen in dit verkorte peptide vervanging van het normaal aanwezige aminozuur door één of meer andere, natuurlijke aminozuren mogelijk is zonder volledig verlies van de werkzaamheid. Dergelijke substituties in het aktieve centrum van het hormoon, de aminozuren 5-10 omvattend, hebben steeds geleid tot veel ingrijpendere verlagingen van de biologische aktiviteit.

Aan ons onderzoek ligt de gedachte ten grondslag, dat inzicht in de specifieke funkties van aminozuren in het aktieve centrum bij de interaktie tussen het hormoon en zijn receptor zou kunnen ontstaan door vervangingen, waarbij een niet-natuurlijk aminozuur wordt geïntroduceerd, dat een zelfde chemische of fysische karakteristiek vertoont als het normaal aanwezige aminozuur.

Uitgaande van deze gedachte is allereerst aandacht besteed aan het aminozuur tryptophaan (positie 9 in ACTH), waarvan bekend is dat het elektron donor eigenschappen bezit die aanzienlijk

groter zijn dan die van alle andere natuurlijke aminozuren. Omdat tevens bekend was dat het niet-natuurlijke pentamethylphenylalanine (Pmp) ongeveer gelijke donor-eigenschappen bezit, zijn substituties door dit aminozuur en door de zeer zwakke elektron donor phenylalanine onderzocht.

Omdat verbindingen met ACTH-aktiviteit tot nu toe steeds ook een melanocyten-stimulerende werking (MSH-aktiviteit) vertoonden is niet alleen het effekt van de substituties op karakteristieke ACTH-werkzaamheid (steroidogenese in de bijnier, lipolyse in vetcellen) onderzocht maar ook op MSH-aktiviteit.

Eenzelfde type onderzoek is gewijd aan arginine (positie 8) waarvan reeds bekend was dat het niet door andere natuurlijke, basische aminozuren (lysine, ornithine) kan worden vervangen. Wij hebben nagegaan of de biologische aktiviteit behouden blijft na substituties, waarbij de sterk basische guanidino funktie in de zijketen behouden blijft, maar de lengte van de zijketen wordt gevarieerd.

De synthese van pentamethylphenylalanine (hoofdstuk II) is uitgevoerd via een bekende procedure. De splitsing van het verkregen racemaat langs enzymatische weg verloopt te traag voor praktisch gebruik. Met behulp van brucine kunnen de L- en D-enantiomeren echter in goede opbrengst zuiver worden verkregen. De natuurlijke L-configuratie kon worden toegewezen aan het rechtsdraaiend isomeer op grond van rotatie-dispersie metingen

aan het vrije aminozuur en het overeenkomstige N-acetyl derivaat, en de, weliswaar langzame, enzymatische hydrolyse van laatstgenoemde verbinding door carboxypeptidase A, die echter niet optrad bij de enantiomeer.

De synthese van de onderzochte ACTH-(1-24) analoga (twee Pmp^9-derivaten in hoofdstuk III en twee Phe^9 derivaten in hoofdstuk IV) is uitgevoerd volgens het schema dat door Schwyzer in de ACTH synthese wordt gebruikt: het tetrapeptide dat de, in ons geval gewijzigde, sequentie 7-10 omvat wordt door successieve fragmentcondensaties met een di- en een tetrapeptide aan het amino uiteinde en een tetradekapeptide aan het carboxyl uiteinde tot het gewenste eindprodukt opgebouwd. De synthese van de tetrapeptiden (7-10) waarvan men uitgaat is in beide gevallen door middel van een fragmentcondensatie verricht. Door gebruik te maken van een nieuwe, alkali-labiele maar uiterst zuur-stabiele amino beschermgroep, de methylsulfonylethyloxycarbonyl group (Msc), waarbij de arginine zijketen onbeschermd wordt gelaten, werd het gewenste tetrapeptide veel sneller en zuiverder verkregen dan bij gebruik van conventionele beschermgroepen. Bij de aminozuuranalyse van de Pmp-derivaten bleek dat pentamethylphenylalanine niet geëlueerd wordt, zoals op grond van het gedrag van het vrije aminozuur verwacht kon worden.

Een overeenkomstige procedure kon uiteraard gebruikt worden voor de synthese van twee α-MSH analoga, omdat de aminozuur sequentie van het 13

aminozuren omvattende α-MSH geheel gelijk is aan die van het amino-eindstandige tridekapeptide uit ACTH. In deze synthese is de Msc groep opnieuw gebruikt, nu voor de zijketen bescherming van lysine op plaats 11. Anders dan bij ACTH is in α-MSH het amino uiteinde geacetyleerd. Wij voerden deze acetyl groep in nadat de synthese van het gehele tridekapeptide amide was voltooid (hoofdstuk V).

Voor een vergelijking van elektron donor eigenschappen tussen overeenkomstige Trp, Pmp en Phe derivaten zijn diverse karakteristieke parameters voor ladingsoverdrachtskomplexen met N,N'-dimethyldipyridylium dichloride (paraquat) onderzocht. Met deze sterke acceptor, die stabiel is in een groot pH interval, kan water als oplosmiddel gebruikt worden (hoofdstuk VI). Uit de resultaten van deze metingen bleek, dat van de gesynthetiseerde ACTH-(1-24) analoga het Pmp^9-derivaat bijna even goede donor-eigenschappen heeft als de natuurlijke Trp^9-verbinding, terwijl het Phe^9-derivaat vrijwel geen donor eigenschappen bezit.

Voor het onderzoek naar de biologische werkzaamheid van de gesynthetiseerde analoga zijn de steroidogenese, lipolytische aktiviteit en melanocyten-stimulerende werking onderzocht (hoofdstuk VII). Van de tetracosapeptiden bleken die met Pmp^9 geen lipolyse, een zeer zwakke steroidogenese maar een verrassend hoge MSH aktiviteit te bezitten. Voor zover bekend is hiermee voor het eerst een duidelijke scheiding tussen ACTH en MSH werking bij een ACTH analogon waargenomen. De over-

eenkomstige Phe^9-peptiden vertonen eveneens een zwakke steroidogenese maar ook een duidelijk afgenomen MSH aktiviteit.
Bij de α-MSH analoga (tridekapeptide amiden) werd geen duidelijk verschil in MSH aktiviteit tussen het Pmp^9-en Phe^9-analogon gevonden.

Omdat deze resultaten vooralsnog geen duidelijke konklusie toelaten over een mogelijke betekenis van Trp^9 in ACTH en α-MSH als elektron donor in de interaktie van deze hormonen met hun receptor, is het interessant te vermelden dat de vervanging van Trp^3 in het hormoon LH-RH (dat het luteïniserend hormoon vrijmaakt) door Pmp, in samenwerking met ons door Coy en medewerkers onderzocht, een produkt oplevert met een zeer hoge LH-RH aktiviteit, terwijl vervanging van Trp^3 door Phe en door diverse andere aminozuren, met zeer zwakke donor eigenschappen, de LH-RH werkzaamheid nagenoeg verloren doet gaan.

Ons onderzoek naar het effekt van vervanging van arginine^8 in ACTH sluit aan bij de reeds bekende substitutie van Arg^8 door een aminozuur met grotere ketenlengte. De invoering van norarginine (een CH_2 groep minder) levert vrij grote synthetische problemen op. De algemene chemische karakteristiek van norarginine verschilt duidelijk van die van arginine (hoofdstuk VIII). Hetzelfde algemene schema volgend als voor de hierboven genoemde analoga is voor de synthese van het benodigde (7-10) tetrapeptide een tweetal wegen gevolgd. In een

hiervan wordt L-α,γ-diaminoboterzuur, dat volgens een aanzienlijk verbeterde en vereenvoudigde methode uit glutaminezuur werd bereid, eerst omgezet in norarginine waarna dit, in de zijketen beschermd door een nitro funktie, in het gewenste peptide werd ingevoerd met behulp van conventionele methoden uit de peptide chemie.
Bij de andere methode werd het diaminoboterzuur zelf ingebouwd en de γ-amino groep pas in het tetrapeptide door amidinering in een guanidino funktie omgezet. Beide methoden leverden een bruikbaar resultaat op.

Uitgaande van het vrije tetrapeptide konden met de conventionele fragmentcondensatie twee ACTH analoga verkregen worden (hoofdstuk IX).
Bij oriënterende biologische aktiviteitsbepalingen *in vivo* met het (1-24)-analogon bleek dat het duidelijk steroidogenese kon induceren. Uit vroeger onderzoek is reeds gebleken dat substitutie van Arg^{8} door homoarginine (een CH_2 groep meer) in ACTH eveneens duidelijk ACTH aktiviteit oplevert.

Curriculum Vitae

De auteur van dit proefschrift is geboren op 6 oktober 1948 te Willemstad, Curaçao (N.A.). Na het behalen van het diploma HBS-B in 1965 aan het Radulphus College aldaar, werd in datzelfde jaar begonnen met de studie in de scheikunde aan de Katholieke Universiteit te Nijmegen. Het kandidaatsexamen (S_2) werd afgelegd in oktober 1968, het doktoraalexamen met als hoofdvak organische chemie (Prof.Dr. R.J.F. Nivard en Dr. G.I. Tesser), de bijvakken biochemie (Prof.Dr. S.L. Bonting en Dr. F.J.M. Daemen) en capita uit de chemie, en de aantekening onderwijsbevoegdheid (Drs. H.A.M. Verkroost) in januari 1971.

Op 1 februari 1971 werd hij aangesteld als wetenschappelijk medewerker aan het Organisch Chemisch Laboratorium. Vanaf 1 mei 1971 is hij als zodanig in dienst van de S.O.N. (Z.W.O.). Het in dit proefschrift beschreven onderzoek werd verricht onder leiding van Prof.Dr. R.J.F. Nivard en Dr. G.I. Tesser.

STELLINGEN

I

De criteria gesteld door Hirschmann aan de "ideale" beschermgroep voor de ϵ-amino funktie van lysine, worden vervuld door de in ons laboratorium ontwikkelde methylsulfonylethyloxycarbonyl groep.

Hirschmann, R. en Veber, D.F. (1973) in The Chemistry of Polypeptides, Essays in honor of Leonidas Zervas (Katsoyannis, P.G., ed.) pp. 125-142, Plenum Press, New York.

II

Voor het bereiden van grote, eventueel gemodificeerde, eiwitfragmenten volgens de semi-synthese procedure, is de werkwijze van Offord overbodig gecompliceerd.

Offord, R.E. (1972) *Biochem. J.* **129**, 499-501.
Offord, R.E. (1973) in Peptides 1972, Proceedings of the 12th European Peptide Symposium (Hanson, H. en Jakubke, H.-D., eds.) pp. 52-56, North-Holland Publishing Co., Amsterdam.
Dyckes, D.F., Creighton, T. en Sheppard, R.C. (1974) *Nature* **247**, 202-203.

III

Het door Hallensleben voorgestelde mechanisme voor de katalyse door elektron acceptoren bij de additie van fenol aan fenylvinylether, is onzinnig.

Hallensleben, M.L. (1971) *Tetrahedron Letters* 3883-3884; *Chem. Ber.* **104**, 3778-3781.

IV

Bij de condensatie van een carboxyl en amino component volgens de methode van Mitin is bij toepassing op nitroarginine en homologen, wanneer deze als carboxyl-eindstandige aminozuren in de reaktie betrokken zijn, de lengte van de zijketen bepalend voor het al of niet verlopen van de beoogde reaktie.

Mitin, Y.V. en Glinskaya, O.V. (1969) *Tetrahedron Letters* 5267-5270.
Mitin, Y.V., Gudkov, A.T., Zapevalova, N.P. en Maximov, E.E. (1973) in Peptides 1972, Proceedings of the 12th European Peptide Symposium (Hanson, H. en Jakubke, H.-D., eds.) pp. 57-64, North-Holland Publishing Co., Amsterdam.
Dit proefschrift.

V

Het vermelden van de vorming van een bijprodukt, b.v. diketopiperazine, bij een nieuwe koppelingsmethode in de peptide chemie, dient vergezeld te gaan van een specificatie van de gebruikte oplosmiddelen.

VI

De resultaten van O'Carra en Jost, die gebaseerd zijn op experimenten met onderling verschillende eiwitten, laten in tegenstelling met de bedoeling van de auteurs in het midden of voornamelijk hydrofobe dan wel ionogene wisselwerking verantwoordelijk is voor de waargenomen niet-specifieke interakties in de affiniteitschromatografie.

O'Carra, P., Barry, S. en Griffin, T. (1974) *FEBS Letters* **43**, 169-175.
Jost, R., Miron, T. en Wilchek, M. (1974) *Biochim. Biophys. Acta* **362**, 75-82.

VII

Het door Oae en medewerkers gegeven mechanisme van de reaktie tussen een selenocarbonyl-N-benzeensulfonylimide en een amine, kan door een eenvoudiger mechanisme worden vervangen.

Tamagaki, S., Sakaki, K. en Oae, S. (1974) *Tetrahedron Letters* 1059-1062.

VIII

Bij het bepalen van de primaire struktuur van polypeptiden volgens de Edman techniek, moet het gebruik van een automaat als enig hulpmiddel als onvoldoende worden gekwalificeerd.

Augusteyn, R.C. en Spector, A. (1971) *Biochem. J.* **124**, 345-355.
Van de Ouderaa, F.J.G. (1974) Proefschrift, Nijmegen.

IX

De publikatie van Robertson en medewerkers over de funktie van sulfhydryl groepen in rhodopsine, draagt weinig bij tot het inzicht in de rol van deze groepen in het molekuul.

Robertson, G.A., Bello, A.C., Stevenson, W.D. en Rockey, J.H. (1974) *Biochem. Biophys. Res. Commun.* **59**, 1151-1156.

X

Gezien de lange periode van voorbereiding van woningbouwprojecten en de geringe flexibiliteit in lopende programma's, kunnen de resultaten van regionale woningmarktonderzoeken, in verband met hun beperkte geldigheidsduur, slechts ten dele ten nutte gemaakt worden.

XI

Ontwerpers van flats zouden moeten worden verplicht eerst enige tijd in een van hun produkten te wonen alvorens nieuwe plannen te mogen indienen.

XII

Gezien de aangekondigde forse prijsverhogingen van gedrukte (lees)boeken, verdient het aanbeveling het gebruik van het goedkopere offset procedé te overwegen.

XIII

In het licht van de toenemende werkloosheid onder academici begint de verhandeling van Erasmus over de wetenschappers en het schrijven van dissertaties een steeds minder schertsmatig karakter te dragen.

Erasmus, D. "Lof der zotheid" pp. 187-195. (Uitg. H.J. Paris N.V., Amsterdam, 1971).

XIV

Het zoeken naar stellingen, die per traditie aan een proefschrift dienen te worden toegevoegd, zou men kunnen beschouwen als een vorm van arbeidstherapie voor de promovendus in een mogelijk ietwat gespannen studiefase tussen het voltooien van het manuscript en de verdediging van het proefschrift.

Nijmegen, 20 december 1974 J.W.F.M. van Nispen